AF/JJW
DAC
FEE

BY HAND DELIVERY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Jin, Hong	Confirmation No.: 8169
Application No.: 09/724,388	Group Art Unit: 1648
Filed: November 28, 2000	Examiner: Lucas, Zachariah
For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES	Attorney Docket No.: 7682-051-999

PETITION UNDER 37 C.F.R. § 1.181 AND M.P.E.P. §§ 1003 ¶6 AND 2307.02

George C. Elliott
Technology Center Director
Technology Center 1600
P.O. BOX 1450
Alexandria, Virginia 22313-1450

Sir:

Pursuant to M.P.E.P. §§ 1003 ¶6 and 2307.02, Petitioners seek revocation of the Final Office Action mailed January 4, 2006 in connection with the above-identified patent application (the "Final Office Action;" attached as Exhibit A). The Final Office Action sets forth rejections that are also applicable to an issued patent (United States Patent No. 6,033,886 to Conzelmann), however, the Final Office Action does not have the required approval by the TC Director. See M.P.E.P. § 2307.02. Petitioners believe the rejections are unfounded and that the Final Office Action should be withdrawn.

The claims pending in this application are identical to those of U.S. Patent 6,033,886 (the '886 patent), and Petitioners seek the declaration of an interference. However, the present pending claims remain rejected, in essence, for lack of enablement under 35 U.S.C. § 112, even though the present application has more disclosure supporting the claims than the '886 patent. Applicants believe that this unfair application of the patent laws can be remedied by declaring an interference between the present application and the '886 patent.

In view of the foregoing, Applicants respectfully request that the Final Office Action be revoked. To expedite the prosecution and allowance of this application, Applicants also present an amendment to narrow the issues. Applicants request that the application be allowed and an interference with United States Patent No. 6,033,886 to Conzelmann be declared.

BACKGROUND

Several of the pending claims, *e.g.*, claims 7 and 13, correspond exactly or substantially to claims 1-19 of United States Patent No. 6,033,886 to Conzelmann, issued March 7, 2000 (the "Conzelmann '886 patent;" attached as Exhibit B).[1] The grounds for rejection in the outstanding Final Office Action include rejections under 37 C.F.R. § 1.112, first paragraph, which are substantially identical to the rejections set forth in the non-final Office Action dated December 28, 2004 (the "Nonfinal Office Action"). These rejections and other issues raised in the Nonfinal Office Action were discussed during an interview held at the U.S. Patent and Trademark Office on September 13, 2005. TC Director Elliott, Interference Specialist Caputa, Examiner Lucas and Supervisory Examiner Housel were present at this interview. As agreed during the interview, Applicants filed a Supplemental Response and a Declaration of Richard R. Spaete under 37 C.F.R. § 1.132 (the "Spaete Declaration") on October 4, 2005, in which all the issues raised in the Nonfinal Office Action were addressed. On January 4, 2006, the Examiner issued a Final Office Action without TC Director approval and without addressing all the facts and arguments set forth in the Supplemental Response and the accompanying Spaete Declaration.

LACK OF TC DIRECTOR'S APPROVAL

Claims copied from the Conzelmann '886 patent were rejected in the Final Office Action. The grounds for these rejections are also applicable to the corresponding claims in the Conzelmann '886 patent. According to M.P.E.P. § 2307.02, a rejection must have the approval of the TC Director if the ground of rejection is also applicable to the corresponding

[1] Applicants informed the U.S. Patent and Trademark Office of this fact in their Response to Restriction Requirement of May 19, 2003.

claims in an issued patent. However, there is no indication in the Final Office Action that any rejection in the Final Office Action was approved by the TC Director.

THE CLAIMS SHOULD BE ALLOWED AND THE INTERFERENCE DECLARED

Applicants provided factual evidence in the form of an expert declaration. The Spaete Declaration sets forth facts that demonstrate that the presently pending claims are enabled. Indeed, the Examiner found Applicants' arguments, which are supported by the Spaete Declaration, persuasive in part. The Examiner appears to agree with the statements of the Spaete Declaration since he did not refute the facts set forth in the Spaete Declaration. However, the Examiner continues to reject the claims under a standard for enablement that is beyond the standard that is usually applied by the U.S. Patent and Trademark Office. In fact, the standard that was applied during the prosecution of the Conzelmann '886 Patent was lower as evidenced by the fact that substantially similar claims were allowed in the Conzelmann '886 Patent although the present application provides more enabling disclosure than the Conzelmann '886 Patent. This unfair application of the patent laws to different applications is the reason for the present Petition. The rejection against the present pending claims should be withdrawn so that the interference can proceed.

The Final Office Action included one new rejection compared to the Nonfinal Office Action.[2] The new rejection was made under 35 U.S.C. § 102 over Calain and Roux, 1993 (J. Virology 67(8):4822-4830; "Calain;" attached as Exhibit C). Applicants believe that this rejection can be overcome by an amendment that clarifies that the compositions allegedly disclosed in Calain do not fall in the scope of the rejected claim. In order to expedite allowance of this application, an amendment and remarks accompanying the amendment are set forth in the attached Amendment under 37 C.F.R. § 1.116 (attached as "Exhibit D").

WITHDRAWN CLAIM 13 SHOULD BE CONSIDERED

Applicants respectfully point out that the rejections against the pending composition and vaccine claims are inapplicable to the method claims. The method claims are directed to a new way of rescuing paramyxoviruses, including wild-type viruses, from plasmid DNA.

---

[2] The new rejection was based on information submitted in an information disclosure statement filed during the period set forth in 37 C.F.R. § 1.97(c). M.P.E.P. § 706.07(a).

These methods can be practiced by the skilled artisan without regard to any modifications that are engineered into the viral genome. Thus, the Examiner's argument that the specification does not provide enough guidance to make paramyxoviruses with certain specified characteristics would not apply to the methods for making paramyxoviruses. In order to expedite prosecution, Applicants would be willing to amend claim 13 to incorporate the limitation of claim 14 that the virus is a paramyxovirus.[3]

Such an amended claim 13 would correspond to claim 17 of the Conzelmann '886 patent. Applicants believe that prosecution of method claim 13 would involve the same prior art searches as the composition claims. In fact, the method of claim 13 is directed to the generation of the compositions of the claims that are presently under consideration. However, since the rejections under 35 U.S.C. § 112, first paragraph, would not apply to claim 13, withdrawal of the restriction as to claim 13 would expedite allowance of a claim that had been copied from the Conzelmann '886 patent.

Because Applicants have shown an intention to claim the subject matter which is claimed in the Conzelmann '886 Patent, an interference should be declared despite the status of claim 13 as withdrawn. See M.P.E.P. § 2303.01. Section 2303.01 of the M.P.E.P. further states that "[t]he statutory requirement of first inventorship should be given primary emphasis and every effort should be made to avoid prematurely issuing a patent where there is an adverse claimant." Based on this policy consideration, the M.P.E.P. states that the examiner should take action toward instituting an interference even if the conflicting claims are withdrawn from consideration in one of the conflicting applications. Accordingly, Applicants request that an interference be declared based on the conflict between claim 13 in the present appliction and claim 17 of the Conzelmann '886 Patent.

Further, in Applicants' amendment of October 4, 2005, which was responsive to the Nonfinal Office Action, Applicants had proposed a claim amendment to withdrawn claim 13.

---

[3] Claims 13-16, which are directed to methods for rescuing virus are withdrawn as a result of the Restriction Requirement (Exhibit E). In particular, these method claims were restricted out because these claims are not limited to paramyxoviruses but instead are directed to non-segmented negative-stranded RNA viruses (see the paragraph spanning pages 2 and 3 of the Restriction Requirement). Claim 14, however, is limited to paramyxoviruses from plasmid DNA.[3] Because the reasoning set forth in the Restriction Requirement does not apply to claim 14, claim 14 and its dependent claims (claims 15 and 16) should be examined together with the pending claims.

The proposed amendment to claim 13 would overcome the reasoning in the Restriction Requirement dated November 19, 2002 as to why claim 13, and the claims dependent thereon, constitute a separate restriction group ("Restriction Requirement;" attached as Exhibit E). The Final Office Action is silent as to Applicants' proposal.

Conclusion

Applicants request that the Final Office Action be revoked, and that the accompanying Amendment and Applicants' proposed amended claim 13 and request for withdrawal of the restriction be entered and considered. Applicants respectfully request that this application be allowed and that an interference with the Conzelmann '886 patent be declared.

The U.S. Patent and Trademark Office is hereby authorized to charge the petition fee under 37 C.F.R. § 1.17(f) in the amount of $400.00 to Jones Day Deposit Account No. 503013. Should any fee be due, please charge the required amount to Jones Day Deposit Account No. 503013.

Respectfully submitted,

Date April 7, 2006

30,742
(Reg. No.)

Laura A. Coruzzi
JONES DAY
222 East 41st Street
New York, New York 10017
Telephone: 212-326-3939

Copies to:
Interference Specialist Anthony C. Caputa (w/ encl)
Supervisory Patent Examiner James C. Housel (w/ encl)
Examiner Zachariah Lucas

Enclosures:
Exhibit A: Office Action dated January 4, 2006
Exhibit B: U.S. Patent No. 6,033,886
Exhibit C: Calain and Roux, 1993, J. Virology 67(8):4822-4830
Exhibit D: Amendment under 37 C.F.R. § 1.116
Exhibit E: Restriction Requirement dated November 19, 2002

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/724,388	11/28/2000	Jin Hong	7682-051-999	8169

20583 7590 01/04/2006

JONES DAY
222 EAST 41ST ST
NEW YORK, NY 10017

EXAMINER	
LUCAS, ZACHARIAH	
ART UNIT	PAPER NUMBER
1648	

DATE MAILED: 01/04/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Appeal and Amendment
Due 4/4/06 (5)

JAN 9 2006
S.Martinek

Office Action Summary	Application No.	Applicant(s)	
	09/724,388	HONG ET AL.	
	Examiner	Art Unit	
	Zachariah Lucas	1648	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *28 June 2005*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *7-10,12-18 and 20-22* is/are pending in the application.

4a) Of the above claim(s) *9 and 13-16* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *7,8,10,12,17,18 and 20-22* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *6-28-05*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Status of the Claims

1. Currently, claims 7-10, 12-18, and 20-22 are pending in the application.

2. In the prior action, mailed on December 28, 2004, claims 7-10, 12-18, and 20-22 were pending, with claims 7, 8, 10, 12, 17, 18, and 20-22 rejected; and claims 9 and 13-16 withdrawn as to non-elected inventions. In the Response filed on October 4, 2005, the Applicant presented additional argument in traversal of the rejections.

3. Claims 7, 8, 10, 12, 17, 18, and 20-22 are under consideration.

Information Disclosure Statement

4. The information disclosure statement (IDS) submitted on June 28, 2005 is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statement has been considered by the examiner.

5. The following reference, cited in the June 2005 IDS, has been crossed out in the reference listing. This is because the reference was previously considered and made of record in the application:

Buchholz et al., J Virol 73: 241-59- cited in the IDS of June 2003.

Claim Rejections - 35 USC § 112

6. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it

pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

7. **(Prior Rejection- Maintained)** Claims 12 and 20 were previously rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement on the basis that the Applicant was not enabled for the making and use of anti-RSV vaccines (or anti paramyxoviral vaccines in general). In Response, the Applicant has submitted arguments, and information in support thereof, indicating that those in the art have successfully developed certain recombinant attenuated RSV viruses as vaccines. These arguments are persuasive in part. However, while the art indicates that RSV vaccines in general may be enabled, there is not sufficient evidence to demonstrate that the Applicant is enabled for the use of any of the claimed viruses as anti-RSV vaccines. As the Applicant notes on page 6 of the Response, there must be a reasonable correlation between the scope of enablement provided in the specification and that which is claimed.

The claims in the present application are drawn to any paramyxoviral, particularly RSV, vaccines wherein virus comprises a genetic modification. The claims indicate that the modification may be any insertion or deletion to the paramyxoviral genome, or any insertion, deletion, or substitution of a complete open reading frame of the genome. As the Applicant has described, the references in the art indicate that specific modifications to the viral genome do result in viruses sufficiently attenuated, but also sufficiently immunogenic, to provide for a protective response in animal models. However, the teachings of these references are also limited to specific modifications. Moreover, the modifications of Karron relate to genetic modifications not within the scope of the current claims (the temperature sensitive variants of that reference are

the result of substitutions, not insertions or deletions, in the genomic sequence). The teachings of the art also indicate that not every modification results in a virus with such a balance between attenuation and immunogenicity. See e.g., the teachings of Tang and Prince (referred to on page 4 of the June 2004 action- which teach that despite a large number of candidate vaccines none had been identified as safe and effective). As has previously been described, the art indicates that there is significant complexity and unpredictability in the making of such attenuated viruses.

While the art indicates that recombinant techniques, such as those described in the present application, permit the construction and evaluation of a broad range of mutations (see e.g., Crowe et al., Vaccine 20(supp 1): S32-37), this does not overcome the inherent unpredictability in determining what modifications would result in viruses comprising sufficient attenuation. Moreover, the Applicant's own teachings indicate that many modifications either have no effect on or increase the operability of viral genes (thus resulting in a virus with no attenuation, or that is more virulent, compared to the wild-type infectious virus), or result in non-viable viruses- neither of which outcomes results in a virus useful as a vaccine. See e.g., App., pages 58-59, and 62-63. Thus, even if the art indicates that the Applicant may be enabled for the use of specific attenuated viruses comprising specific attenuating mutations as anti-RSV vaccines, it does not demonstrate that mere ability to produce viral attenuations automatically enables those in the art to produce any anti-RSV, or any anti-paramyxoviral, vaccine without undue experimentation. The methods described merely permit those in the art to run the numerous trials required to identify such viruses.

In addition, the present application provides little guidance as to full scope of modifications that may be made. While the application does disclose certain modifications to the

L protein, and describes rescued virus comprising deletions of certain genes, there is no demonstration that the disclosed viruses result in sufficiently attenuated viruses for use as vaccines. Further, the modifications disclosed with respect to the L protein fall outside the scope of the present claims as they identify substitutions, and not insertions or deletions. It is also noted that the claims read on insertions or deletions of nucleotides, or the insertion, deletion, or substitution of any open reading frame, in any gene from any paramyxovirus genome. While the application suggests regions in the genomes where attenuating mutations may be attempted, there is no identification of specific positions or modifications that would result in an attenuated phenotype. Thus, the claims are drawn to a broad genus of attenuated viruses, the scope of which those in the art would be required to identify for themselves.

In view of the breadth of the claims, the unpredictability in the art, and amount of experimentation that would be required to practice the full scope of the claims, and the comparatively limited guidance presented, the application is not found enabling for any vaccine according to the rejected claims for the reasons above, and for the reasons of record. Thus, while the Applicant's arguments are found persuasive in part, they are not deemed sufficient to demonstrate that the application has provided sufficient enabling support to reasonable correlate to the scope of what is claimed.

8. **(Prior Rejection- Maintained)** Claims 7, 8, 10-12, 17, 18, 20, and 21 were rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to

make and/or use the invention. The claims read on genetically manipulated replication competent infectious RSV comprising any insertion or deletion, or any insertion, substitution, or deletion of an entire open reading frame (ORF).

The Applicant traverses this rejection on the basis of their assertion that they have provided an enabling disclosure, and that the experimentation required to identify the claims viruses in the present application is not undue. In particular, the Applicant asserts that there is no requirement for a "reasonable certainty before performing" an experiment that it will succeed so long as there is a reasonable amount of guidance with respect to the direction of the experimentation.

As previously indicated, the Wands case put forth a series of factors to be considered in making a determination as to whether an application is enabling for a claimed invention. The factors that may be considered include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. The Applicant asserts that the Wand decision indicates that there is no requirement for a reasonable certainty that the experiment would be successful a priori, so long as a reasonable amount of guidance has been presented. The Applicant then continues on the assert that the application teaches methods for the modification and screening of modified virus, and provides adequate guidance with respect to the direction of experimentation.

The Wands case related to the production of antibodies against a particular antigen that were able to bind to the antigen with high affinity. The court determined that the Applicant was

enabled for methods of using such antibodies as the application disclosed a method for the production of such antibodies, in combination with a number of working examples. It is noted that the court characterized the experiment for identifying the antibodies as the entire process of immunizing an animal, forming hybridomas, and screening the hybridomas for antibodies with the required affinity. 8 U.S.P.Q. 2d, at 1407. The court also noted that "Wands carried out this entire procedure three times, and was successful each time in making at least one antibody that satisfied all of the claim limitations." Id. Based on this characterization, the court determined that the teachings of the application enabled the scope of the claimed invention- methods of using any antibody with the indicated level of affinity for the identified antigen. Thus, the Wands case involved a situation in which a single experiment with no variable factors was repeated, and where an operable embodiment resulted in each instance.

In the present case, the claims are broadly drawn to genetically modified paramyxoviruses comprising any insertion or deletion. The application provides several suggestions as to the types and placement of modifications that may be made to the viral genomes, and provides examples of experiments that resulted in both operational and in non-functional viruses. See e.g., pages 58-59. Unlike the experimentation involved in the Wands case, each experiment in the present instance involves the production of a single specific viral mutant, and the testing of this specific mutant to determine if it is indeed replication competent and infectious. If one looks to RSV alone, with a genome of over 15,000 nucleotide bases, this permits a large number of potential insertions and deletions, each of which represents a different variable and experiment to be performed.

The Applicant asserts that they have provided guidance as to where to make the modifications. In particular, the Response notes several suggestions as to the types of modifications that may be made. For example, the Response indicates that "non-coding regulatory regions can be modified to obtain attenuated viruses," and that "modifications can be introduced into the viral surface antigens to interfere with the binding affinity of the virus to the host cell." Response, page 13. However, while the application provides such general suggestions, the is no guidance as to what specific modifications can be made to each of the various paramyxoviral sequences that would result in the claimed viruses. Such specific guidance is, however, required as was demonstrated on pages 58-59 which illustrates that different modifications to the same region of the viral genome can have very different effects- resulting unpredictably in both viruses within or without the claimed genus. Thus, the present case is drawn to a broad genus in an uncertain art, and for which comparatively few working examples have been provided. Further, those working examples which have been provided provide little information as to the operability of modifications outside of the L gene, or even to the operability of other modifications within this gene. Further, unlike the wands case which involved relatively little experimentation in that the exact same process was repeatedly performed, the practice of the full scope of the invention in the present case requires that those in the art perform repeated experiments with numerous variables (the different potential modifications) with little guidance as to the specific modifications that would result in virus according to the claims.

The Applicant further asserts that the *Angstadt* case supports their conclusion that the application provides enabling support for the claimed genus. In particular, the Applicant asserts that the decision held the claims at issue therein enabled on the grounds that the skilled artisan

would have read the inventor's specification for directions in making the compounds at issue, and would then determine if such compounds had been made. However, this is not the sole basis on which the Court found enablement in that case. The *Angstadt* court repeated referred to the forty successful experiments of the inventor in that case, also noting that only one of the tested compounds was not effective. 190 U.S.P.Q. 214, at 218. Further, the court also noted that the experiments required to test the various substances were not complex or complicated. Thus, unlike the present case which does involve complex manipulation of genes, in which a large number of inoperative embodiments have been shown, and for which there is little predictability with reference to other potential manipulations, and for the reasons indicated in the prior action, the Applicant reliance on *Angstadt* does not appear well founded.

Further, these same facts relating to the present case indicate that, given the scope of the present claims, the application has not provided sufficient guidance to enable those in the art to make or use viruses according to the rejected claims without undue experimentation. For these reasons, and for the reasons of record, the rejection is maintained.

9. **(Prior Rejection- Maintained)** Claims 7, 8, 10-12, 17, 18, and 20 were rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Applicant asserts that where a specification discloses any relevant identifying characteristics, a rejection for lack of written

description is misplaced. The Applicant asserts that sufficient identification has been presented in the present case.

In support of their position, the Application presents three arguments. First, the Applicant asserts that they have provided a function, and means to test for the presence of the functions. Second, the Applicant asserts that they have provided examples of the claimed genus. Finally, the Applicant asserts that they have provided both a functional requirement and a structural limitation on the claimed genus. These arguments are not found persuasive.

The claims are drawn to compositions comprising any genetically manipulated paramyxovirus that is both infectious and replication competent, wherein the virus comprises a genome that comprises either an insertion or a deletion. It is noted that the respiratory syncytial virus, the subject virus of claim 10, comprises a genome of over 15,000 nucleotide bases. App., page 3. The claims broadly read on a genus wherein the virus may comprise an insertion or a deletion anywhere within this genomic sequence.

With respect to the first argument in traversal, the Applicant argues that in the instant case, the specification teaches examples of how to identify operative viruses, and teaches methods of testing for the required functions. See, Response, page 15. However, while the application may disclose methods of identifying which of the many potential mutations to the genome would result in a virus with the required functions (infectivity and replication competence), it is settled that disclosure of a method of identifying a compound does not provide descriptive support of the compound itself. See e.g., *University of Rochester v. Searle & Co.*, 69 USPQ2d 1886, at 1895 (CA FC 2004). Thus, the Applicant's assertion that they provides such

methods is not found a persuasive argument that the application provides descriptive support for viruses that may be so identified.

The second argument in traversal is that the Examiner has improperly relied on the decision of *In re Smythe* to support the position that disclosure of a sufficient number of working examples fails to provide written descriptive support for a claimed invention. The Applicant responds that this decision actually indicated that a disclosure of a limited number of species might provide such support. The Applicant's interpretation of the case is only partially correct. However, as was indicated in the prior action, in *Smythe* the CCPA specifically indicated that such support for a claimed genus may not be found from the disclosure of a number of species "where there is unpredictability in performance of certain species or subcombinations other than those specifically enumerated." *In re Smyth*, 178 U.S.P.Q. 279 at 284-85 (CCPA 1973). As has been previously described, the art and the application in the present case demonstrate that there is such uncertainty in the operability of species other than those specifically disclosed. This is exemplified by the applications disclosure of the varying results of individual modifications to the L gene on pages 58-59, and 62-63. As the application demonstrates that modifications at various positions among the viral genome would result have various effects on the operability of any particular species, and as the application fails to teach what structures and sequences throughout the RSV genome correlate to the viral functions of infectivity and replication competence, the disclosure of the operative species fails to provide adequate support for the whole of the claimed genus of RSV variants, much less the variants of any paramyxovirus.

It is noted that Applicant discloses that the N, P, and L proteins are the minimal requirements for viral replication competence. Page 6. However, the present claims include

modifications to these proteins, without a description in the application identifying those specific portions of these proteins that must be maintained to result in an infectious particle. Thus, for the reasons indicated above, and previously of record, the disclosure of these minimal genes required for operability is not found sufficient to provide descriptive support as the application does not provides support for the full scope of modifications that may be made to these proteins without a loss of their functions, and therefore of operability of the virus as whole.

The Applicant's third argument is they have provided both a function and an associated structure as is required by the *Eli Lilly* case cited in the prior action. Specifically, the Applicant asserts that descriptive support for the claimed genus is found in the description of the claimed viruses as being replication competent and infectious, and as having the structural limitation that it is a virus of a paramyxoviridae family comprising a "certain modification." This argument is not found persuasive.

It is first noted the claims are not directed to viruses with "a certain modification," but is directed to viruses with certain types of modifications. This is an important distinction because the application does disclose certain specific modifications that may be made to the RSV genome that would appear to result in infectious and replication competent viruses. However, the claims are not limited to these specific modifications. What the claims describe are paramyxoviruses with any modification of a certain type to the viral genome- e.g., an insertion or a deletion of one or more bases in the viral genome.

The argument is not found persuasive with respect to the claimed genus of viruses because the indicated structures do not correlate with the required functions. The *Eli Lilly* case does not merely suggest the presence of a structure and a function, but indicates that where the

Applicant wishes to rely on a function, the functional characteristic may be used in combination with a known or disclosed correlation between the function and a structure. *Eli Lilly*, 43 USPQ2d at 1406. In the present case, there has been no demonstration that there is any correlation between the functional limitations and the mere presence of a viral gene with any particular type of genetic modification. Again, this is exemplified by the disclosure relating to the disclosed modifications to the L protein, where modifications of the same type, but at different positions in the RSV L gene had different functional effects. The fact that similar modifications at different positions resulted in genomes with different functional characteristics demonstrates that the structural limitations of having a paramyxoviral genome with a certain type of limitation does not a structurally identify genomes of viruses capable of replication and infection. Rather, the teachings indicate that viruses that have the required functions merely have different genomic modifications from those that are not replication competent, infectious, or both. Nor is there any structural identifier presented by which those in the art could distinguish between hypothetical genomes of viruses that do have these functions from those that do not. The Applicant's third argument in traversal is therefore also not found persuasive.

Similarly, with respect to claims 18 and 20-22, while the application teaches that the N, P, and L genes are required for viral replication, the application does not disclose the minimal genes required for an infectious and replication competent virus. For example, the application indicates on page 3 that the F and G proteins are involved in viral attachment and cell entry. However there is no disclosure as to whether such proteins would therefore be required components, either in their native form or as a substitute from another strain of the same virus (e.g. substitution of a F or G from type A for that in a type B genome) are required for the

functional limitations. While the application discloses the deletion of the SH and M2-1 genes, such does not indicate what other genes may be deleted without a loss of the required functions.

For these reasons, and for the reasons of record, the rejection is maintained.

Claim Rejections - 35 USC § 102

10. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

11. **(New Rejection- Reference cited in IDS)** Claim 7 is rejected under 35 U.S.C. 102(b) as being anticipated by Calain et al. (J Virol 67: 4822-30- cited in the IDS of June 2005). This claim is directed to a genetically manipulated, replication competent, and infectious paramyxoviridae virus wherein the virus comprises a insertion into the genomic sequence. Calain teaches a recombinant Sendai virus (a paramxyovirus) comprising an insertion of six bases. See e.g., page 4825, Table 2 (the derivative identified as the -17 derivative). The reference teaches that the RNA of this derivative sequence is capable of replication (page 4825, right column), and that the polynucleotides resulted in competent virus particles when the plasmids where replicated by a helper virus. Pages 4826-27 (disclosing that viruses expressed from the plasmid in the presence of a helper virus resulted in "competent" virus, i.e. virus that were infective and replication competent). Thus, the reference teaches a genetically manipulated rabies virus comprising an

insertion and which is both infectious and replication competent. The reference therefore anticipates the indicated claim.

Claim Rejections - 35 USC § 103

12. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

13. **(Prior Rejection- Maintained)** Claims 18, and 20-22 were rejected under 35 U.S.C. 102(e) as being anticipated by Murphy et al., U.S. Patent 5,993,824 (of record in the action mailed on September 24, 2003). The rejection is withdrawn from claims 18 and 20. However, the rejection is maintained over claim 21, and claim 22 to the extent that it depends from claim 21. The Applicant traverses the rejection by asserting that the present application is entitled to priority to the '439 application, which predates the Murphy reference.

The Applicant asserts that support for the presently claimed inventions may be found in columns 43, 16, and 47 of U.S. Patent 5,840,520, which issued from the '439 application. However, while the patent teaches the deletion of all of the genes and inserting a CAT gene in their place, this does not result in an infectious and replication competent virus as is claimed. Additionally, while the parent application refers to the deletion of genes in influenza, and suggests that these teachings may be applied to RSV, there are no teachings specifically directing those in the art to deletion of the M2-2 ORF of RSV. Finally, it is not clear how the teachings

relating to substitution of one F or G gene for another provide support for the deletion of the M2-2 RSV gene. Nor does there appear to be any other support in the parent application for the species of the claimed RSV comprising a deletion of the M2-2 ORF. It is noted that disclosure of a genus does not provide descriptive support for an undisclosed species. Thus, while the patent/prior application indicates that deletions may be made, there are no teachings relating to the deletion of the M2-2 ORF to make a recombinant, infectious, and replication competent virus.

Conclusion

14. No claims are allowed.

15. Applicant's submission of an information disclosure statement under 37 CFR 1.97(c) with the fee set forth in 37 CFR 1.17(p) on June 28, 2005 prompted the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 609.04(b). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event,

however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

16. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Zachariah Lucas whose telephone number is 571-272-0905. The examiner can normally be reached on Monday-Friday, 8 am to 4:30 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Housel can be reached on 571-272-0902. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Z. Lucas
Patent Examiner

James C. Housel 12/26/05

LIST OF REFERENCES CITED BY APPLICANT

(Use several sheets if necessary)

ATTY DOCKET NO.	APPLICATION NO.
7682-051-999	09/724,388
APPLICANT: Hong Jin *et al.*	
FILING DATE: November 28, 2000	GROUP: 1648

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
32	DP	EP 94202089.2	7/18/94	EP				
32	DQ	EP 0 780 475 B1	6/9/99	EP				

OTHER REFERENCES *(Including Author, Title, Date, Pertinent Pages, Etc.)*

32	DR	Baer et al., 1990, Virology, 2nd ed., Fields et al., eds., Raven Press Ltd., New York, pp. 883, 887
32	DS	Ballart (Eschle) et al., 1991, "RETRACTION: Infectious measles virus from cloned cDNA," EMBO J. 10(11):3558
32	DT	Ballart et al., 1990, "Infectious measles virus from cloned cDNA," EMBO J. 9(2):379-384
32	DU	Belshe et al., 1992, "Evaluation of a live attenuated, cold-adapted parainfluenza virus type 3 vaccine in children," J. Clin. Microbiol. 30(8):2064-2070
	DV	Buchholz et al., 1999, "Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter," J. Virol. 73(1):251-259
32	DW	Calain et al., 1993, "The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA," J. Virol. 67(8):4822-4830
32	DX	Calain et al., 1992, "Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA," Virol. 191:62-71
32	DY	Crowe et al., 1996, "Acquisition of the ts phenotype by a chemically mutagenized cold-passaged human respiratory syncytial virus vaccine candidate results from the acquisition of a single mutation in the polymerase (L) gene," Virus Genes 13(3):269-273
32	DZ	Deng et al., 1991, "High-efficiency protein synthesis from T7 RNA polymerase transcripts in 3T3 fibroblasts," Gene 109(2):193-201
32	EA	Dimock and Collins, 1993, "Rescue of synthetic analogs of genomic RNA and replicative-intermediate RNA of human parainfluenza virus type 3," J. Virol. 67(5):2772-2778
32	EB	Elroy-Stein and Moss, 1990, "Cytoplasmic expression system based on constitutive synthesis of bacteriophage T7 RNA polymerase in mammalian cells," Proc. Natl. Acad. Sci. USA 87(17):6743-6747
32	EC	Fields et al., 1996, Virology 106, 168
32	ED	Fuerst et al., 1986, "Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase," Proc. Natl. Acad. Sci. USA 83(21):8122-8126
32	EE	Gallione & Rose, 1985, "A single amino acid substitution in a hydrophobic domain causes temperature-sensitive cell-surface transport of a mutant viral glycoprotein," J. Virol. 54(2):374-382
32	EF	Garcin et al., 1995, "A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus," EMBO J. 14(24):6087-6094
32	EG	Kingsbury, ed., 1991, "Deletion mutants of paramyxoviruses," in: The Paramyxoviruses, Plenum Press, New York, pp. 275-298
32	EH	Kolakofsky et al., 1998, Paramyxovirus RNA synthesis and the requirement for hexamer genome length: The Rule of Six revisited," J. Virol.. 72(2):891-899
32	EI	Kucera et al., 1985, "Pathways of the early propagation of virulent and avirulent rabies strains from the eye to the brain," J. Virol. 55(1):158-162
32	EJ	Lafay et al., 1994, "Vaccination against rabies: construction and characterization of SAG2, a double avirulent derivative of SADBern," Vaccine 12(4):317-320

12/20/05

32	EK	Li et al., 1988, "Site-specific mutations in vectors that express antigenic and temperature-sensitive phenotypes of the M gene of vesicular stomatitis virus," J. Virol. 62(10):3729-3737
32	EL	Lieber et al., 1989, "High level gene expression in mammalian cells by a nuclear T7-phase RNA polymerase," Nucleic Acids Res. 17(21):8485-8493
32	EM	Ligas et al., 1988, "A herpes simplex virus mutant in which glycoprotein D sequences are replaced by b-galactosidase sequences binds to but is unable to penetrate into cells," J. Virol. 62(5):1486-1494
32	EN	Morita et al., 1987, "Phenotypic revertants of temperature-sensitive M protein mutants of vesicular stomatitis virus: sequence analysis and functional characterization," J. Virol. 61(2):256-263
32	EO	Owens et al., 1993, "Cytoplasmic domain requirement for incorporation of a foreign envelope protein into vesicular stomatitis virus," J. Virol. 67(1):360-365
32	EP	Pattnaik, 1992, "Infectious defective interfering particles of VSV from transcripts of a cDNA clone," Cell 69:1011-1020
32	EQ	Radecke et al., 1997, "Reverse Genetics Meets the Nonsegmented Negative-Strand RNA Viruses," Rev. Med. Virol. 7(1):49-63
32	ER	Rauh et al., 1991, "Pseudorablies virus gllycoproteins gII and gp50 are essential for virus penetration," J. Virol. 65(10):5348-5356
32	ES	Schnell et al., 1994, "Infectious rabies virus from cloned cDNA," Ninth Int'l Conference on Negative Strand Viruses (October 2-7, 1994) pp. 87, Abstract 90
32	ET	Schnitzer et al., 1979, "Morphological and biochemical characterization of viral particles produced by the tsO45 mutant of vesicular stomatitis virus at restrictive temperature," J. Virol. 29(1):185-195
32	EU	Seif et al., 1985, "Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein," J. Virol. 53(3):926-934
32	EV	Shioda et al., 1986, "Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN and L proteins," Nucleic Acids Res. 14(4):1545-1563
32	EW	Takeda et al., 2000, "Recovery of pathogenic measles virus from cloned cDNA," J. Virol. 74(14):6643-6647
32	EX	Tordo et al., 1992, "Evolution of negative stranded RNA genomes," Seminars in Virol. 3:341-357
32	EY	Whetter et al., 1994, "Analysis of hepatitis A virus translation in a T7 polymerase-expressing cell line," Arch. Virol. Suppl. 9:291-298
32	EZ	Whitt et al., 1990, "A fusion-defective mutant of the vesicular stomatitis virus glycoprotein," J. Virol. 64(10):4907-4913
32	FA	Whitt et al., 1989, "Glycoprotein cytoplasmic domain sequences required for rescue of a vesicular stomatitis virus glycoprotein mutant," J. Virol. 63(9):3569-3578
32	FB	Wyatt et al., 1995, "Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells," Virology 210(1):202-205

EXAMINER	**DATE CONSIDERED** 12/20/05

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Notice of References Cited	Application/Control No. 09/724,388	Applicant(s)/Patent Under Reexamination HONG ET AL.	
	Examiner Zachariah Lucas	Art Unit 1648	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Crowe et al., Respiratory syncytial virus vaccine development, Vaccine, Volume 20 Supp 1, Pages S32-S37 (October 2001).
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

ELSEVIER

Vaccine 20 (2002) S32–S37

Vaccine

www.elsevier.com/locate/vaccine

Respiratory syncytial virus vaccine development

James E. Crowe Jr.*

Departments of Pediatrics and Microbiology and Immunology, Vanderbilt University Medical Center, D-7235 Medical Center North, 1161 21st Avenue South, Nashville, TN 37232-2581, USA

Abstract

Development of an RSV vaccine for infants has been hindered by the lack of an ideal animal model that exhibits disease, and the challenge of effectively immunizing very young infants who are immunologically immature. Nevertheless, significant progress has been made recently in developing live attenuated viruses and protein subunit vaccine candidates. Numerous vaccine candidates are currently in early clinical trials. This paper reviews the significant obstacles to development of RSV vaccines, and the progress made to date.

Keywords: Respiratory syncytial virus (RSV); Parainfluenza virus (PIV); Vaccines

1. Obstacles in the development of RSV vaccines

RSV vaccine development began over three decades ago, yet there is still no licensed vaccine. The development and testing of vaccine candidates is problematic because of limitations in the experimental systems and unique obstacles inherent in the epidemiology of the disease (see Table 1). Preclinical testing of vaccine candidates is inhibited by the lack of an ideal animal model. Infection of numerous mouse strains has been studied because of the wealth of immunologic reagents available, yet mice are not fully permissive for RSV replication [1,2]. Cotton rats are more susceptible to infection [3], but the lack of cotton rat immunologic reagents is a limitation and the high body temperature (>39°C) can lead to overestimation of the level of attenuation of live temperature sensitive (ts) vaccine candidates. Studies in non-human primate such as monkeys and chimpanzees have given critical preclinical results that allowed progression to human trials and predicted the level of replication of vaccine viruses in those trials [4–6]. Nevertheless, these animals still are not as permissive to RSV infection as are human infants. Ultimately, definitive studies of the safety, genetic stability, immunogenicity, and protective efficacy of RSV vaccine candidates can only be carried out in humans. The target age group for immunization is quite young, since the age of peak incidence of severe disease is 6–8 weeks. Infants in the first several weeks of life are immunologically immature, and transplacental maternally derived IgG antibodies suppress the primary antibody response. Lung growth and significant changes in airway resistance occur rapidly in the first months of life. The high airway resistance of neonates at the bronchiolar level leaves them at high-risk of expiratory airway obstruction during RSV infection. Therefore, evaluation of candidate vaccines in humans must be carried out with great care, in safety trials starting in adults, then progressing in a step-wise manner to seropositive children and infants, and finally to seronegative or RSV-naïve children and infants [4,6]. This testing strategy requires tremendous clinical effort and research expertise.

Another obstacle is that different target populations for RSV immunization vary widely in immunologic maturity and experience with previous RSV infection. The majority of hospitalizations caused by RSV infection occur in very young infants who are RSV-naïve but possess varied levels of maternally-acquired serum antibodies. Most infants who are hospitalized have no identifiable risk factors for severe disease that distinguish them from other infants of the same age [7,8]. Therefore, the main target population for immunization is all normal infants in the first month(s) of life. Other populations at risk beyond the neonatal period include premature infants, and patients with congenital heart disease or chronic lung disease (especially bronchopulmonary dysplasia) [8], and the elderly [9]. One vaccine candidate or strategy may not be optimal for each of the target populations. We have shown in safety trials that live attenuated viruses that are not infectious or immunogenic in adults or older children can replicate to high levels and cause respiratory disease in very young infants [4,6].

Immunization of neonates is difficult because they exhibit a significant immunologic immaturity that may dictate the use of a multi-dose vaccine strategy in the first weeks of

* Tel.: +1-615-343-8064; fax: +1-615-343-9723.
E-mail address: james.crowe@vanderbilt.edu (J.E. Crowe Jr.).

0264-410X/01/$ – see front matter
PII: S0264-410X(01)00287-0

Table 1
Obstacles to RSV vaccine development

Animal models are not fully permissive for infection
Requirement for bivalent vaccine (two antigenic subgroups)
Young age of primary vaccine target population
Special safety concern when immunizing neonates
Immunologic immaturity of neonatal target population
Maternal antibodies inhibit immune responses in the target population
Disease occurs at the portal of entry (the respiratory mucosa)
Mucosal immunity to respiratory viruses is short-lived and incomplete
FI-RSV vaccine caused enhanced disease

life. Safety concerns are significant at this age. Although maternal antibodies against RSV may afford partial protection to the infant for the first few weeks of life, these antibodies inhibit antibody responses to immunization. We have demonstrated this suppression in experimental infections with live attenuated vaccine candidates [4,6], and in animal model systems such as rodents and primates [10,11]. Antibody-mediated immune suppression also has been observed in epidemiological studies of wild-type RSV infection [7,12].

Mucosal immunity to respiratory viruses is short-lived and incomplete, therefore, sterilizing immunity against infection is not a realistic goal. For example, adults with recent RSV infection can be reinfected in the upper respiratory tract multiple times in serial fashion with the same virus suspension [13]. Protection against lower respiratory tract disease during RSV infection is a more feasible goal, since naturally-acquired wild-type virus infection of infants appears to provide protection against lower respiratory tract disease [14,15].

The complications of the formalin-inactivated RSV (FI-RSV) vaccine studies in the 1960s have had a chilling effect on the development and testing of RSV vaccine candidates. Vaccinees that received FI-RSV immunization experienced increased rates of severe lower respiratory tract disease [16,17]. Much effort has been invested in attempts to define the immune mechanisms underlying the enhanced immunopathology induced by FI-RSV. The testing of RSV subunit vaccines in particular has been complicated by the finding of enhanced histopathology in rodent models [18]. Although non-replicating antigens may not be the appropriate vaccine candidates for RSV-naïve infants, consideration has been made for using these vaccines as booster immunizations for previously infected high-risk children [19,20], the elderly [21], and for maternal immunization [22].

2. Immunity to RSV infection and disease

Cytolytic T-lymphocytes (CTLs) are likely the principal mediators of resolution of acute infection [23]. The peak level of CTLs coincides with viral clearance during primary infection in experimental animals, and animals or patients lacking T cells exhibit prolonged RSV shedding [24,25]. CTLs probably do not usually contribute significantly to protection against reinfection because of the short-lived nature of the activated CTL response. CTL precursors certainly persist in the host, but activated CTLs are short-lived [26].

Antibodies are the principal mediators of resistance to RSV reinfection. Secretory and serum antibodies appear to protect against RSV disease and infection [27–29]. Serum RSV IgG antibodies protect the lower respiratory tract against infection but have little impact on upper respiratory tract virus replication. In the upper tract, local antibodies including secretory IgA appear to play a major role in protection. Interestingly, the IgA induced by infection or immunization of infants do not appear to neutralize virus in classical in vitro virus neutralization assays [6,30] but do correlate with protection of the upper respiratory tract [6,29]. Protective antibodies are directed to the fusion (F) and attachment (G) surface glycoproteins, therefore, most investigators have studied the response to these proteins. Numerous assays have been developed to describe the quality and quantity of RSV antibodies induced by immunization. Infection induces much higher titers of antibodies that bind to RSV F or G proteins in ELISA than that neutralize RSV in vitro. Subsets of RSV-specific antibodies neutralize virus and a smaller subset of these antibodies inhibit virus-mediated fusion in vitro. Such neutralizing antibodies appear to be the key to protection against reinfection.

3. Vaccine development

3.1. Formalin-inactivated RSV (FI-RSV) vaccine trials

Clinical trials conducted in the 1960s using a FI-RSV preparation caused severe enhanced disease in immunized infants during subsequent natural infection [16,17]. The rationale for using this vaccine at that time was that inactivated viruses were thought to carry little risk of reactogenicity. Inactivated vaccines for poliovirus and influenza virus were known to be safe and efficacious. We may never truly know the mediators of enhanced disease in those particular children, however, it is clear that experimental FI-RSV preparations prime for a Th-2 like cytokine response in the lungs of rodents that upon subsequent infection is associated with cellular infiltration with low levels of virus-specific CTL activity [24]. FI-RSV likely primed for increased CD4+ T cell-mediated airway inflammation without inducing a normal CTL or protective antibody response. Plans for testing RSV vaccine candidates in humans must always address this potential serious complication of enhanced disease.

3.2. Live virus vaccines

Live virus infection of the nasopharynx induces a balanced immune response similar to that induced by natural infection, and therefore, is unlikely to induce enhanced disease. Enhanced disease has not been observed during

reinfection with wild-type viruses or experimental live attenuated virus immunizations. Topical infection with attenuated viruses also is an attractive strategy because it induces both secretory and serum antibodies, and can infect subjects even in the presence of maternal antibodies [6]. The first generation of live attenuated RSV vaccine candidates was generated by isolating mutant viruses generated by passage in cell culture, using selection based on in vitro phenotypes correlated with in vivo attenuation. RSV mutant viruses that were cold-passaged (cp) or ts (i.e. grow well at the permissive temperatures of the nasopharynx but not at the higher temperatures of the lungs) were isolated, biologically cloned, and tested in adults, children and infants [31]. These initial viruses were attenuated, infectious, and immunogenic in older infants, but exhibited potential problems with genetic stability (a low level of loss of the in vitro phenotype during virus shedding in human vaccines). The clinical relevance of the low level of instability was not established, but testing of live attenuated viruses was discontinued for a number of years as other strategies were explored. Obviously, it is desirable to use attenuated viruses with a high level of genetic stability of the principal attenuating mutations. However, absolute fidelity of replication in susceptible subjects of every base of the genome of these RNA viruses, whose polymerases lack proofreading activity, is probably not feasible or necessary. Our efforts to generate a second generation of biologically derived live attenuated respiratory virus vaccine candidates have demonstrated that attenuated RNA viruses can be identified that exhibit a high level of genetic stability in humans. The enhanced stability of these viruses appear to be associated with the presence of multiple attenuating mutations of differing classes (specifying ts, host-range, or cold-adapted phenotypes).

About 10 years ago, we began work to generate more genetically stable live attenuated RSV vaccine candidates based on the principle that viruses with multiple attenuating mutations are more genetically stable than viruses with single mutations. Starting with the previously tested host-range mutant cp-RSV and introducing a series of multiple ts mutations by chemical mutagenesis resulted in the generation of large panels of promising vaccine candidates. We found that viruses that possess the cp-RSV attenuating mutations and at least two ts mutations were highly attenuated and genetically stable in rodents, chimpanzees, and humans, including seronegative infants. At least four such mutants have been examined to date in Phase I clinical trials in seronegative infants [4,6]. Current efforts are centered on identifying candidates that are sufficiently attenuated and immunogenic in the very youngest infants (birth to age 2 months).

3.3. Live vaccines derived via genetic engineering

In the mid-1990s, scientists at the National Institutes of Health, USA, developed molecular cloning techniques that allow for the generation of live RSV viruses from plasmid DNA copies (cDNA) of the virus genome (see Fig. 1)

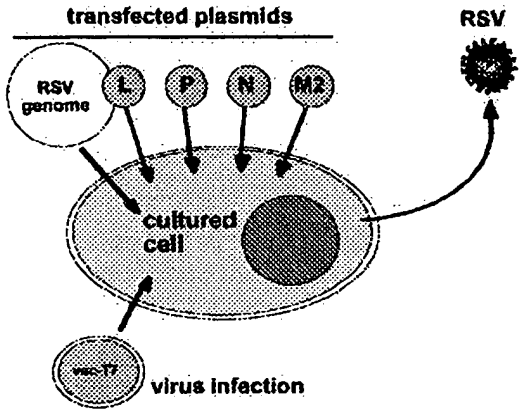


Fig. 1. Schematic representing the generation of live RSV by recombinant means. Four genes essential for RSV replication (*L, P, N, M2*) are expressed from plasmids encoding DNA copies of the genes, with expression driven by bacteriophage T7 polymerase supplied by a co-infected recombinant vaccinia virus. The expressed RSV proteins drive the replication of RSV genome supplied by a simultaneously transfected full-length plasmid DNA copy, generated by recombinant means [32]. Similar systems are used for other paramyxoviruses.

[32]. Now, site-directed mutations can be engineered into viruses, allowing the rational development of candidate vaccines. Novel strategies are being explored, such as combinations of point mutations, gene insertions, gene deletions, and chimeric viruses. First, we determined the genetic basis of attenuation of many of the biologically derived vaccine candidates by nucleotide sequence analysis of cDNA. The ts phenotype of these mutants was encoded in most cases by single nucleotide changes in open-reading frames or in virus regulatory regions such as the gene-start region [33,34]. This preliminary work allowed for the introduction of additional mutations from a menu of known attenuating mutations to achieve step-wise increases in attenuation. Multiple mutations from separate biologically derived ts mutants have now been combined into novel viruses that are created in the laboratory from cDNA. Other interesting strategies are possible. Immunomodulation is being investigated using co-expression of cytokines (for ex interleukin-2) by recombinant RSV [35]. Gene deletions offer the possibility of causing an attenuation phenotype that is unlikely to be lost by single point mutations elsewhere in the residual genome. Recombinant RSV that does not express the NS1 or M2-2 protein is highly attenuated and immunogenic in chimpanzees [36]. Remarkably, chimeric paramyxoviruses that utilize a mixture of proteins from different viruses can be generated and propagated. A relatively conservative chimeric virus was made by changing the RSV subgroup through replacement of the F and G surface glycoproteins of RSV subgroup A with those of subgroup B to generate chimeric live attenuated RSV subgroup B vaccine candidates. [37]. More ambitious has been the replacement of surface proteins using those from related viruses, such as a parainfluenza virus (PIV) candidate vaccine containing the hemagglutinin-neuraminidase

and fusion glycoproteins of PIV-1 and the remaining proteins from PIV-3 [38]. This strategy can be used to achieve a modified Jennerian approach, such as the engineering of human RSV surface proteins on a bovine RSV strain [39], or conversely, a single bovine gene (nucleoprotein) from a bovine PIV-3 into a human PIV-3 virus [40]. Human PIV-3 expressing the hemagglutinin protein of measles virus provides a potential method for immunization against both measles virus and PIV-3 [41]. Obviously, such vaccines require careful evaluation because of the possibility of altered tropism, but with a step-wise cautious preclinical and clinical approach such chimeric viruses could be advanced.

3.4. Virus vector delivery systems

Live virus vectors that express RSV F and G gene products have been tested in rodents and non-human primates. The vectors that have been examined are poxviruses such as vaccinia virus and modified vaccinia Ankara, and adenovirus [42,43]. Vaccinia viruses that express F (vac-F) or G (vac-G) were immunogenic and protective in rodents. We found however, that immunization with these vaccine candidates was less immunogenic in chimpanzees and did not protect the lower respiratory tract of these animals against wild-type RSV challenge. The use of vaccinia virus vectors in infants also faces significant safety concerns as well, and these viruses are unlikely to undergo clinical trials. Newer poxvirus vectors expressing RSV proteins that do not produce infectious virus progeny in humans could be considered for use in humans [44]. The evaluation of adenovirus recombinant viruses in animals failed to induce adequate level of protection against RSV [43].

3.5. Subunit vaccines

Protective antibodies against RSV are directed to the F and G proteins, and therefore, purified F and G proteins have been studied as vaccine candidates. F and G protein vaccines have been prepared as purified proteins from various expression systems using mammalian cells, insect cells, or bacteria. Whole purified F and G protein preparations have been isolated from RSV-infected mammalian cell culture using immunoaffinity or other chromatographic means. These purified proteins have been evaluated extensively in preclinical models and clinical trials. Studies in rodents suggested that these subunit vaccine preparations may induce an altered T cell response, similar to that induced by FI-RSV, when used as the primary immunogen in RSV-naïve subjects [45,46]. In addition, these subunit products are not particularly immunogenic in young infants. Therefore, these preparations are not likely to be good candidates for immunization of very young infants. However, these purified proteins are well tolerated and moderately immunogenic at a 50 μg dose in older children and adults. These vaccines may be suitable for immunization of previously infected patients who are at high-risk of severe disease during reinfection, such as children with bronchopulmonary dysplasia or cystic fibrosis, or the elderly [47–49]. These vaccines have also been considered for use in older children with asthma. In addition, such vaccines are being tested in maternal immunization strategies. Lastly, subunit vaccines could be used in combination with live attenuated vaccine candidates. The live attenuated cpts-248/404 RSV vaccine has been evaluated in combination with a subunit RSV vaccine (PFP-2) in healthy young and older adults [50].

A second strategy for RSV subunit immunization more recently developed is the expression of a portion of the G glycoprotein in bacteria. The BB2GNa recombinant derived protein contains a conserved polypeptide from RSV G protein fused to a streptococcal albumin-binding protein [51]. This subunit vaccine is immunogenic and protective in rodent studies, and adult safety studies are underway.

4. Plasmid DNA vaccines

The rationale for this approach for RSV immunization of infants is that protective antigens might be expressed in infants for a prolonged period. DNA vaccines therefore, might be able to immunize the infant at the appropriate time point at which waning maternal antibodies have fallen below suppressive levels in the infant. One study suggested that F encoded in a plasmid could induce a protective response in rodents [52]. The feasibility of this approach for use in neonatal humans is unclear at this time.

5. Other approaches

The classical Jennerian approach of using animal strains of RSV as vaccine candidates in humans was studied [53], but the protective antigens of these viruses are antigenically distinct from human RSV. Other preclinical approaches include the use of peptides encoding antigenic epitopes, and antigens incorporated in immune stimulating complex formulations (ISCOMS) [54,55]. The use of new adjuvants to augment or alter the type of immune response to RSV vaccine candidates, especially subunit vaccines, has also been investigated in animal models [56,57].

Acknowledgements

This work was supported by grants from the NIH (USA) (NICHD HD36311, NIDR DE14039) and the NIH Respiratory Pathogens Research Unit N01-AI-65298.

References

[1] Prince GA, Horswood RL, Berndt J, Suffin SC, Chanock RM. Respiratory syncytial virus infection in inbred mice. Infect Immunol 1979;26:764–6.

[2] Graham BS, Perkins MD, Wright PF, Karzon DT. Primary respiratory syncytial virus infection in mice. J Med Virol 1988;26:153–62.
[3] Prince GA, Jenson AB, Horswood RL, Camargo E, Chanock RM. The pathogenesis of respiratory syncytial virus infection in cotton rats. Am J Pathol 1978;93:771–9.
[4] Karron RA, Wright PF, Crowe Jr. JE, et al. Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial virus vaccines in chimpanzees and in human adults, infants, and children. J Infect Dis 1997;176:1428–36.
[5] Crowe Jr. JE, Bui PT, Firestone C-Y, et al. Live subgroup B respiratory syncytial virus vaccines that are attenuated, genetically stable, and immunogenic in rodents and non-human primates. J Infect Dis 1996;173:829–39.
[6] Wright PF, Karron RA, Belshe RB, et al. Evaluation of a live, cold-passaged temperature-sensitive, respiratory syncytial virus (RSV) vaccine in infancy, J Infect Dis 2000;182:1331–42.
[7] Glezen WP, Paredes A, Allison JE, Taber LH, Frank AL. Risk of respiratory syncytial virus infection for infants from low-income families in relationship to age, sex, ethnic group, and maternal antibody level. J Pediatr 1981;98:708–15.
[8] Hall CB, Kopelman AE, Douglas RG, Geiman JM, Meagher MP. Neonatal respiratory syncytial virus infection. N Engl J Med 1979;300:393–6.
[9] Falsey AR, Treanor JJ, Betts RF, Walsh EE. Viral respiratory infections in the institutionalized elderly: clinical and epidemiologic findings. J Am Ger Soc 1992;109:203–8.
[10] Crowe Jr JE, Firestone C-Y, Murphy BR. Passively-acquired antibodies suppress humoral but not cell-mediated immunity in mice immunized with live attenuated RSV vaccines. J Immunol, 2001, in press.
[11] Crowe Jr JE, Bui PT, Siber GR, Elkins WR, Chanock RM, Murphy BR . Cold-passaged, temperature sensitive mutants of human respiratory syncytial virus (RSV) are highly attenuated, immunogenic, and protective in seronegative chimpanzees, even when RSV antibodies are infused shortly before immunization. Vaccine 1995;13: 847–55
[12] Englund JA. Passive protection against respiratory syncytial virus disease in infants: the role of maternal antibody. Pediatr Infect Dis J 1994;13:449–53.
[13] Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis 1991;163:693–8.
[14] Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. Am J Dis Child 1986;140:543–6.
[15] Kasel JA, Walsh EE, Frank AL, Baxter BD, Taber LH, Glezen WP. Relation of serum antibody to glycoproteins of respiratory syncytial virus with immunity to infection in children. Viral Immunol 1987;1:199–205.
[16] Kim HW, Canchola JG, Brandt CD, et al. Respiratory syncytial virus disease in infants despite poor administration of antigenic inactivated vaccine. Am J Epidemiol 1969;89:422–34.
[17] Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Steward CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol 1969;89:405–21.
[18] Graham BS. Pathogenesis of respiratory syncytial virus vaccine-augmented pathology. Am J Respir Crit Care Med 1995;152:S63–6.
[19] Piedra PA, Grace S, Jewell A, et al. Sequential annual administration of purified fusion protein vaccine against respiratory syncytial virus in children with cystic fibrosis. Pediatr Infect Dis J 1998;17:217–24.
[20] Groothuis JR, King SJ, Hogerman DA, Paradiso PR, Simoes EAF. Safety and immunogenicity of a purified F protein respiratory syncytial virus (PFP-2) vaccine in seropositive children with bronchopulmonary dysplasia. J Infect Dis 1998;177:467–9.
[21] Falsey AR, Walsh EE. Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in ambulatory adults over age 60. Vaccine 1996;14:1214–8.
[22] Englund JA, Glezen WP, Piedra PA. Maternal immunization against viral disease. Vaccine 1998;16:1456–63.
[23] Cannon MJ, Openshaw PJM, Askonas BA. Cytotoxic T cells clear virus but augments lung pathology in mice infected with respiratory syncytial virus. J Exp Med 1988;168:1163–8.
[24] King Jr JC, Burke AR, Clemens JD, Nair P, Farley JJ, et al. Respiratory syncytial virus illnesses in human immunodeficiency virus and non-infected children. Pediatr Infect Dis J, 1993;12:733–9.
[25] Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T-lymphocyte subsets in the pathogenesis of primary infection and re-challenge with respiratory syncytial virus in mice. J Clin Invest 1991;88:1026.
[26] Connors M, Collins PL, Firestone C-Y, Murphy BR. Respiratory syncytial virus (RSV) F, G, M2 (22K), and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. J Virol 1991;65:1634–7.
[27] Muelanaer PM, Henderson FW, Hemming VG, et al. Group-specific serum antibody responses in children with primary and recurrent respiratory syncytial virus infections. J Infect Dis 1991;164:15–21.
[28] Groothius JR, Simoes EAF, Levin MJ, et al. Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. N Engl J Med 1993;329:1524–30.
[29] Mills V J, Van Kirk JE, Wright PF, Chanock RM. Experimental respiratory syncytial virus infection in adults. Possible mechanisms of resistance to infection and illness. J Immunol 1971;107:123–30.
[30] McIntosh KHB, Orr I, Chao RK, Barkin RM. The immunologic response to infection with respiratory syncytial virus in infants. J Infect Dis 1978;138:24–32.
[31] Wright PF, Shinozoki T, Fleet W, et al. Evaluation of a live, attenuated recombinant respiratory syncytial virus vaccine in infants. J Pediatr 1976;88:931–6.
[32] Collins PL, Hill MG, Camargo E, Grosfeld H, Chanock RM, Murphy BR. Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open-reading frame of the *M2* mRNA in gene expression and provides a capability for vaccine development. Proc Natl Acad Sci USA 1995;92:11563–7.
[33] Crowe Jr JE, Firestone C-Y, Collins PL, Murphy BR. Acquisition of the ts phenotype by a chemically mutagenized cold-passaged human respiratory syncytial virus vaccine candidate results from the acquisition of a single mutation in the polymerase *L* gene. Virus Genes 1996;13:271–5.
[34] Firestone C-Y, Whitehead S, Collins PL, Murphy BR, Crowe Jr JE. Nucleotide sequence analysis of the respiratory syncytial virus (RSV) subgroup A cold-passaged (cp), temperature sensitive (ts) cpts-248/404 live attenuated virus vaccine candidate. Virology 1996;225:419–22.
[35] Bukreyev A, Whitehead SS, Prussin C, Murphy BR, Collins PL. Effect of co-expression of interleukin-2 by recombinant respiratory syncytial virus on virus replication, immunogenicity, and production of other cytokines. J Virol 2000;74:7151–7.
[36] Teng MN, Whitehead SS, Bermingham A, et al. Recombinant respiratory syncytial virus that does not express the NS1 or M2-2 protein is highly attenuated and immunogenic in chimpanzees. J Virol 2000;74:9317–21.
[37] Whitehead SS, Hill MG, Firestone C-Y, et al. Replacement of the F and G proteins of respiratory syncytial virus (RSV) subgroup A with those of subgroup B generates chimeric live attenuated RSV subgroup B vaccine candidates. J Virol 1999;73:9773–80.
[38] Tao T, Davoodi F, Cho CJ, et al. A live attenuated recombinant chimeric parainfluenza virus (PIV) candidate vaccine containing the hemagglutinin-neuraminidase and fusion glycoproteins of PIV-1 and the remaining proteins from PIV-3 induces resistance to PIV-1 even in animals immune to PIV-3. Vaccine 2000;18:1359–66.
[39] Buchholz UJ, Granzow H, Schuldt K, Whitehead SS, Murphy BR, Collins PL. Chimeric bovine respiratory syncytial virus with glycoprotein gene substitutions from human respiratory

syncytial virus (HRSV): effects on host-range and evaluation as a live-attenuated HRSV vaccine. J Virol 2000;74:1187–99.
[40] Bailly JE, McAuliffe JM, Durbin AP, Elkins WR, Collins PL, Murphy BR. A recombinant human parainfluenza virus type 3 (PIV-3) in which the nucleocapsid N protein has been replaced by that of bovine PIV-3 is attenuated in primates. J Virol 2000;74:3188–95.
[41] Durbin AP, Skiadopoulos MH, McAuliffe JM, et al. Human parainfluenza virus type 3 (PIV-3) expressing the hemagglutinin protein of measles virus provides a potential method for immunization against measles virus and PIV-3 in early infancy. J Virol 2000;74:6821–31.
[42] Crowe Jr JE, Collins PL, London WT, Chanock RM, Murphy BR. A comparison in chimpanzees of the immunogenicity and efficacy of live attenuated respiratory syncytial virus (RSV) temperature-sensitive mutant vaccines and vaccinia virus recombinants that express the surface glycoproteins of RSV. Vaccine 1993;11:1395–404.
[43] Hsu KH, Lubeck MD, Davis AR, et al. Immunogenicity of recombinant adenovirus-respiratory syncytial virus vaccines with adenovirus types 4, 5, and 7 vectors in dogs and a chimpanzee. J Infect Dis 1992;166:769–75.
[44] Wyatt LS, Whitehead SS, Venanzi KA, Murphy BR, Moss B. Priming and boosting immunity to respiratory syncytial virus by recombinant replication defective vaccinia virus MVA. Vaccine 1999;18:392–7.
[45] Murphy BR, Sotnikov AV, Lawrence LA, Banks SM, Prince GA. Enhanced pulmonary histopathology is observed in cotton rats immunized with formalin-inactivated respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV 3–6 months after immunization. Vaccine 1990;8:497–502.
[46] Connors M, Collins PL, Firestone C-Y, et al. Cotton rats previously immunized with a chimeric RSV FG glycoprotein develop enhanced pulmonary pathology when infected with RSV, a phenomenon not encountered following immunization with vaccinia RSV recombinants or RSV. Vaccine 1992;10:475–84.
[47] Groothuis JR, King SJ, Hogerman DA, Paradiso PR, Simoes EA. Safety and immunogenicity of a purified F protein respiratory syncytial virus (PFP-2) vaccine in seropositive children with bronchopulmonary dysplasia. J Infect Dis 1998;177:467–9.
[48] Piedra PA, Grace S, Jewell A, et al. Purified fusion protein vaccine protects against lower respiratory tract illness during respiratory syncytial virus season in children with cystic fibrosis. Pediatr Infect Dis J 1996;15:23–31.
[49] Falsey AR, Walsh EE. Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in the institutionalized elderly. Vaccine 1997;15:1130–2.
[50] Gonzalez IM, Karron RA, Eichelberger M, et al. Evaluation of the live attenuated cpts-248/404 RSV vaccine in combination with a subunit RSV vaccine (PFP-2) in healthy young and older adults. Vaccine 2000;18:1763–72.
[51] Power UF, Plotnicky-Gilquin H, Huss T, et al. Induction of protective immunity in rodents by vaccination with a prokaryotically expressed recombinant fusion protein containing a respiratory syncytial virus G protein fragment. Virology 1997;230:155–66.
[52] Li X, Sambhara S, Li CX, et al. Protection against respiratory syncytial virus infection by DNA immunization. J Exp Med, 1998;188:681–8.
[53] Piazza FM, Johnson SA, Darnell ME, Porter DD, Hemming VG, Prince GA. Bovine respiratory syncytial virus protects cotton rats against human respiratory syncytial virus infection. J Virol 1993;67:1503–10.
[54] Chargelegue D, Obeid OE, Hse SC, et al. A peptide mimic of a protective epitope of respiratory syncytial virus selected from a combinatorial library induces virus-neutralizing antibodies and reduces viral load in vivo. J Virol. 1998;72:2040–6.
[55] Hu KF, Elvander M, Merza M, Akerblom L, Brandenburg A, Morein B. The immunostimulating complex (ISCOM) is an efficient mucosal delivery system for respiratory syncytial virus (RSV) envelope antigens inducing high local and systemic antibody responses. Clin Exp Immunol 1998;113:235–43.
[56] Hancock GE, Speelman DJ, Frenchick PJ, Mineo-Kuhn MM, Baggs RB, Hahn DJ. Formulation of the purified fusion protein of respiratory syncytial virus with the saponin QS-21 induces protective immune responses in BALB/c mice that are similar to those generated by experimental infection. Vaccine 1995;13:391–400.
[57] Neuzil KM, Johnson JE, Tang YW, et al. Adjuvants influence the quantitative and qualitative immune response in BALB/c mice immunized with respiratory syncytial virus FG subunit vaccine. Vaccine 1997;15:525–32.

JOURNAL OF VIROLOGY, Aug. 1993, p. 4822–4830
0022-538X/93/084822-09$02.00/0

Vol. 67, No. 8

The Rule of Six, a Basic Feature for Efficient Replication of Sendai Virus Defective Interfering RNA

PHILIPPE CALAIN AND LAURENT ROUX*

Department of Genetics and Microbiology, University of Geneva Medical School, C.M.U., 9 avenue de Champel, 1211 Geneva 4, Switzerland

Received 23 February 1993/Accepted 8 May 1993

The addition of the hepatitis delta virus genomic ribozyme to the 3′ end sequence of a Sendai virus defective interfering RNA (DI-H4) allowed the reproducible and efficient replication of this RNA by the viral functions expressed from cloned genes when the DI RNA was synthesized from plasmid. Limited nucleotide additions or deletions (+7 to −7 nucleotides) in the DI RNA sequence were then made at five different sites, and the different RNA derivatives were tested for their abilities to replicate. Efficient replication was observed only when the total nucleotide number was conserved, regardless of the modifications, or when the addition of a total of 6 nucleotides was made. The replicated RNAs were shown to be properly enveloped into virus particles. It is concluded that, to form a proper template for efficient replication, the Sendai virus RNA must contain a total number of nucleotides which is a multiple of 6. This was interpreted as the need for the nucleocapsid protein to contact exactly 6 nucleotides.

Sendai virus (SV) is a member of the *Paramyxoviridae* family and the genus *Parainfluenza*. It is a nonsegmented negative-stranded RNA virus, a murine correspondent of the human respiratory parainfluenza type 1 to 4 viruses, and a close cousin of mumps virus and measles virus, and it is more distantly related to human respiratory syncytial virus. The viral RNA of this family of viruses is more than 15,000 nucleotides long, complementary to the mRNA sense, and noninfectious. The minimum infectious unit is in fact the viral RNA wrapped in a helical nucleocapsid structure by about 2,600 copies of the NP protein and associated with 300 copies of the P/C and 40 copies of the L proteins (14), the latter two of which form the RNA polymerase complex (for a recent review, see reference 10). Because the basic rules governing encapsidation and replication are unclear, and also because of the large size of the viral RNA, it has not been possible up to now to produce infectious virus particles by transfecting cells with a plasmid expressing the viral RNA. Therefore, genetic manipulation of the viral genome is not yet possible for this category of viruses, consequently limiting the study of the viral functions and the development of other applications (e.g., vaccines).

In recent years, however, successful encapsidation and replication of shorter versions of the viral RNA synthesized from plasmids have been achieved by using viral functions expressed from cloned viral genes or provided by regular infections with the homologous virus (3, 4, 16, 19, 20). Two approaches have previously been taken for designing the shorter viral RNAs. On one hand, the RNA sequence has been constructed by flanking the reporter chloramphenicol acetyltransferase (CAT) gene sequence with the 3′ and 5′ viral RNA end sequences (4, 16, 19). On the other hand, the viral RNA was simply a copy of a defective interfering (DI) RNA naturally selected for its ability to efficiently replicate (3, 20). These two approaches obeyed different strategies. The former approach relied on the extreme sensitivity of the enzymatic assay performed by the reporter gene to detect replication. The latter approach relied on the ensured presence of all of the required sequences (some of which may have been omitted from the synthetic constructs) needed for efficient replication, thereby also ensuring that the reactions involved would be closer to those taking place in natural infections.

These systems, more than showing that proper encapsidation and replication of an RNA expressed from plasmid were feasible, opened the possibility of dissecting the viral functions as well as the *cis* RNA sequences involved. In this study, after we had observed that large internal deletions in the DI-H4 RNA obliterated its replication by the SV functions provided from plasmids, we constructed RNA derivatives with limited nucleotide modifications. From the replication pattern of the derivatives emerged the need for the total length of the RNA to be a multiple of 6 nucleotides. This conclusion is in agreement with the total number of nucleotides in the SV nondefective and DI-H4 RNAs and is consistent with the conclusions of previous structural studies of the nucleocapsid.

MATERIALS AND METHODS

Viruses and cells. SV Harris strain nondefective and DI-H4 copy-back defective viral stocks were prepared and characterized as described before (23). Vaccinia recombinant virus expressing the T7 RNA polymerase, vTF7-3, a gift from Bernard Moss (National Institutes of Health, Bethesda, Md.), has been described by Fuerst and colleagues (9) and was used accordingly. vTF7-3 stocks were prepared in Vero cells with titers ranging from 5×10^7 to 5×10^8 PFU/ml. CV1 and Vero cells were routinely grown in minimal Eagle medium supplemented with 5% fetal calf serum under a 5% CO_2 atmosphere.

Sequences and plasmids. The total SV RNA sequence is derived from the work of Shioda and colleagues (24, 25). It contains a total of 15,384 nucleotides. The pGem plasmids expressing the SV NP, P/C, and L genes under the control of the T7 RNA polymerase have been described previously (5, 12). The cloning and the description of the pSP65 plasmid containing the SV DI-H4 insert under the control of the T7 RNA polymerase promoter, pSV-DIH4/A, have been de-

* Corresponding author.

scribed by Calain et al. (3). In summary, this plasmid contains the sequence of the DI-H4 copy-back RNA (1,410 nucleotides long; see Results) under the control of the T7 RNA polymerase promoter. After linearization of the plasmid by *Bsm*I, the T7 RNA transcript represents an exact copy of the plus-strand DI-H4 RNA (3). Addition of the hepatitis delta virus genomic ribozyme (21, 22) to the end of the T7 RNA transcript was done in the following way. pSV-DIH4/A was digested with *Bsm*I, treated with Klenow to remove the 3' protruding nucleotides, and finally digested with *Bam*HI flanking the *Bsm*I site to generate a blunted-*Bam*HI pSV-DIH4/A (3). The hepatitis delta virus genomic ribozyme sequence was recovered by polymerase chain reaction (PCR) amplification from plasmid HN 3-24 (27), a gift from Michael Lai (Los Angeles, Calif.), with primer Rib1 (5' GGCCGGCATGGTCCC 3') complementary to the ribozyme 5' end and primer Rib3 (5' CGAGGATCCGTC CCATTCGCCATTAC 3') complementary to the 3' ribozyme end sequence and flanked with a *Bam*HI site. The PCR product was digested with *Bam*HI and cloned into pSV-DIH4/A blunted-*Bam*HI (see above). The resulting plasmid was called pSV-DIH4/Rbz. Large deletions in the insert of pSV-DIH4/Rbz were generated by excisions of *Eco*RV (positions 958 and 1244), *Hpa*I-*Eco*RV (positions 461, 958, and 1244), or *Spe*I-*Eco*RV (positions 125, 153, 958, and 1244) fragment, followed by fill-in reactions, when adequate, and religations. The resulting plasmids were called, respectively, pSV-DIH4/RbzΔ1125, pSV-DIH4/RbzΔ628, and pSV-DIH4/RbzΔ296, with the last number referring to the resulting size of the DI-H4 insert (see Fig. 2). Limited stepwise modifications were created by opening pSV-DIH4/Rbz at unique sites (see Table 2). After treatment with 1 U of T4 DNA polymerase in standard buffer for 15 min at 12°C in the presence of 0.3 mM deoxynucleotide triphosphate (dNTP), the plasmids were religated. Every modification was verified by sequencing.

Ribozyme activity testing. To test the self-cleaving activity of the hepatitis delta virus ribozyme, the plasmids were linearized at the *Bam*HI site located 85 nucleotides downstream from the ribozyme cleavage site (these 85 nucleotides in fact represent the ribozyme sequence attached to the *Bam*HI site). The T7 RNA transcripts were then synthesized in the presence of [α-^{32}P]UTP in vitro with the T7 RNA polymerase (Biofinex, Praroman, Switzerland) according to the manufacturer's instructions. The labelled transcripts were then ethanol precipitated and separated on a 6% polyacrylamide gel to detect the 85-nucleotide fragment resulting from the self-cleavage activity. Negative controls for cleavage were made by cutting the pSV-DIH4/Rbz plasmids with *Bgl*I, which cleaves in the middle (38 nucleotides from the 5' end) of the ribozyme sequence. The dried gels were quantitated in a phosphorimager (Molecular Dynamics) to estimate the percentage of cleavage.

Encapsidation-replication assays. For a complete replication assay, CV1 cells, seeded on 9-cm-diameter petri dishes, were infected with vTF7-3 at a multiplicity of infection of 2 to 3. One hour postinfection, the cells were transfected with pGem4-NP (5 μg), pGem4-P/C (5 μg), pGem4-L (1.5 μg), and pSV-DIH4/Rbz or its derivatives (5 μg) as previously described (3, 5). Cytoplasmic extracts were prepared 24 h postinfection, and CsCl gradient-purified nucleocapsid RNAs were analyzed by Northern (RNA) blotting (18). Replication was monitored by using a 5' ex riboprobe (18) of positive polarity (same polarity as the T7-RNA transcript). Encapsidation was investigated in an assay from which pGem4-P/C and pGem4-L were omitted. The putative nucleocapsids were then purified and the nucleocapsid RNAs were analyzed by Northern blotting with a 5' ex riboprobe of negative polarity (complementary to the T7 RNA transcripts [18]).

In vitro-synthesized T7 and SP6 RNA transcripts. In vitro RNA transcripts used as size and polarity markers were synthesized by T7 RNA or SP6 RNA polymerase (Biofinex), according to the supplier's instructions after proper linearization of pSV-DIH4 plasmids devoid of the ribozyme sequence (see Results).

Preparation of SV DI viral stocks from the DI nucleocapsids rescued from plasmids. The DI-H4 (or its derivative) nucleocapsids obtained in a replication assay were purified by banding on CsCl gradients, concentrated by centrifugation through glycerol onto a cushion of 68% sucrose in D_2O (SW55; 40,000 rpm, 12°C, 3 h), and transfected with 25 μl of TransfectACE (GIBCO BRL) into CV1 cells previously infected (2 h) with nondefective SV (multiplicity of infection = 20). After 20 h of infection, performed in the presence of 100 μg of cytosine arabinoside per ml to block contaminating vaccinia virus multiplication, the cells were recovered by 5 mM EDTA treatment and injected into 9-day-old embryonated chicken eggs (about 3×10^6 cells per egg). After 3 days of incubation at 33°C, the allantoic fluids (AFs) were collected and clarified by centrifugation (45 min, 12,000 × *g*). One milliliter of the AFs was mixed with 20 PFU of nondefective SV per cell and used to infect CV1 cells. The presence of infectious DI nucleocapsids originating from pSV-DI-H4 plasmids amplified in the CV1 cells was finally monitored by Northern blot and reverse PCR analysis of the intracellular viral nucleocapsid RNAs (see Fig. 5 for a schematic representation of the complete protocol).

PCR analysis. The viral nucleocapsids produced in the CV1 cells infected with the AFs obtained from the embryonated eggs (see above) were purified on a CsCl gradient. The nucleocapsid RNAs (isolated from about 5×10^6 cells) were then reverse transcribed with 20 pmol of the oligomer SV-LN6738 (15265-5'-TTCTGCACGATAGGGAC-3'-15248, with the numbers on each end referring to the position in the SV genome) of negative polarity with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in 50-μl reaction mixtures according to the supplier's instructions. The cDNAs were diluted 1:10 in H_2O, and 5 μl was amplified with 20 pmol of oligomers SV-LN6738 and SV-B1 (14894-5'-GTTGAAGACAATTTC TAGAAGACT-3'-14917) as amplimers in a 100-μl reaction mixture containing 2.5 U of AmpliTaq DNA polymerase (Cetus)–3 mM Mg^{2+}–50 μM dNTP in a GeneAmp PCR System 9600 according to the supplier's instructions (94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, 30 cycles). PCR amplifications of reverse transcriptase minus reactions were performed to control for contaminating plasmid DNA (see Results and Fig. 6).

RESULTS

Replication of DI-H4 RNA starting from plasmid pSV-DIH4/Rbz. We have previously shown that a T7 RNA transcript made from plasmid pSV-DIH4/A was successfully encapsidated and replicated by the SV functions NP, P, and L provided in *trans* by cotransfection with pGem plasmids (3). In those experiments, the correct 3' extremity of the RNA transcript was generated by *Bsm*I linearization of pSV-DIH4/A before transfection. This method of transfection is generally viewed as a low-efficiency transfection method, and accordingly, this was reflected in the poor

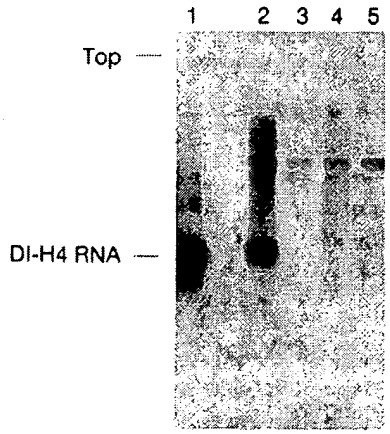


FIG. 1. Replication of DI-H4 RNA from plasmid pSV-DIH4/Rbz. About 10 million CV1 cells were infected with vTF7-3 and transfected with pSV-DIH4/Rbz (lanes 2 and 3) or pSV-DIH4/A (lanes 4 and 5) either with pGem-NP, -P/C, and -L (lanes 2 and 4) or without these viral replicating functions (lanes 3 and 5). Twenty-four hours posttransfection, the nucleocapsids were purified and the viral RNA was analyzed by Northern blotting as described in Materials and Methods. The 5′ ex riboprobe used is of positive polarity. In lane 1, 1/200th of a similar amount of CV1 cells infected with the SV viral stock DI-H4, in the presence of vTF7-3, is similarly analyzed. The molecular weight marker indicating the size of the natural DI-H4 RNA is shown (lane 1).

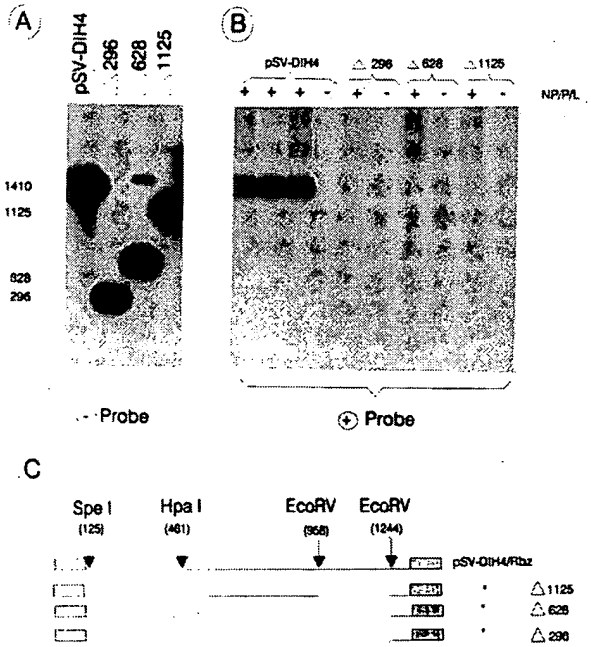


FIG. 2. Unsuccessful replication of DI-H4 RNAs with large internal deletions. (A) In vitro T7 RNA transcripts made from plasmids pSV-DIH4/A, pSV-DIH4-Δ1125, pSV-DIH4-Δ628, and pSV-DIH4-Δ296. (B) Nucleocapsid-purified RNAs isolated from replication assays after transfections with pSV-DIH4/Rbz, pSV-DIH4/Rbz-Δ296, pSV-DIH4/Rbz-Δ628, and pSV-DIH4/Rbz-Δ1125, with or without the viral replicating functions. Transfections with pSV-DIH4/Rbz are presented in triplicate. In panels A and B, the RNAs are analyzed in identical Northern blots and the 5′ ex riboprobes are of negative and positive polarities, respectively. (C) Schematic description of the deletions. Shaded boxes represent inverted repeats of the DI RNAs. The numbers in the designations of the deleted plasmids refer to the final size of the DI RNA sequence.

reproducibility of the reaction as well as in the efficiency of replication, which was often at the limit of Northern blot detection (3). To avoid such a drawback, the self-cleaving ribozyme sequence of the hepatitis delta virus was cloned into the pSV-DIH4/A to generate plasmid pSV-DIH4/Rbz. Replication assays using pSV-DIH4/Rbz to provide the DI-H4 RNA were indeed found to be more consistent and efficient. A typical example is presented in Fig. 1, in which the results of assays using pSV-DIH4/Rbz or pSV-DIH4/A are shown side by side. Under conditions in which transfection with pSV-DIH4/Rbz allowed the detection of a strong replication signal (lane 2), the use of *Bsm*I-linearized pSV-DIH4/A (lane 4) showed no signal above the background (lane 5) at this level of gel exposure. Although the efficiency of replication varied by factors of 2 to 5 from experiment to experiment, in more than 15 different experiments no failure to replicate DI-H4 RNA from pSV-DIH4/Rbz was ever observed. In these experiments, the criteria for assessing replication are identical to those used earlier (3), i.e., (i) the RNA was purified in the form of a nucleocapsid banding at the right density in a CsCl gradient and is resistant to ribonuclease digestion (not shown), (ii) it is complementary to the T7 RNA transcript (the riboprobe used in the Northern blot is of the same polarity as this transcript), and (iii) its presence depended on the cotransfection of all three SV replicating functions (Fig. 1, lane 3).

Large internal deletions in DI-H4 sequence obliterate replication. Confident that the replication assay was very reproducible, we made large internal deletions in the DI-H4 sequence in an attempt to estimate the effect of total RNA size on the efficiency of replication. As shown in Fig. 2C, the internal deletions left untouched the ends of the RNAs; even the inverted repeats of 110 nucleotides were conserved. Surprisingly, none of the deleted RNAs was replicated in conditions in which integral DI-H4 RNA was replicated (Fig. 2B). As shown in Fig. 2A, the deleted plasmids were as competent as pSV-DIH4 for synthesizing the T7 transcripts. Moreover, no reduction of the self-cleaving activity used to generate the exact 3′ end of the transcript could be observed (Table 1).

Effects of limited internal deletions in DI-H4 RNA on replication. Explanations to account for the failure in replication reported above could be, among others, the removal

TABLE 1. Self-cleaving activities of large DI-H4 RNA deletions[a]

Derivative of pSV-DIH4/Rbz	Arbitrary U of radioactive signal in:		Size of 85-mer (%)[b]	Amt of radioactivity in 85-mer (%)[c]	% Cleavage[d]
	Top bands	85-mer			
	633,970	9,586	5.40	1.50	28
-1125	1,020,019	20,318	6.60	2.00	30
-628	560,712	18,759	11.30	3.30	29
-296	123,370	12,982	21.40	10.50	49

[a] For details on the procedure used to estimate self-cleaving activity, see Materials and Methods.
[b] Relative to that of the total transcript.
[c] Relative to that in the total transcript.
[d] Determined by dividing amount of radioactivity in 85-mer by size of 85-mer.

TABLE 2. Replication ability of DI-H4 RNA and its derivatives[a]

Plasmid or derivative	Sites (429) Cel II	(980) BstX1	(1026) Mun I	(1068) Sau I	Sau I*	(1239) Dra III	Total base modification	Replication	Encapsidation	Position in six phase
Nb. of bases changed	+3	-4	+4	+3	+2	-3				
pSV-DIH4/Rbz	–	–	–	–	–	–	None	+	+	0
pSV-DIH4/Rbz										
-01	+	–	–	–	–	–	+3	–	ND[b]	+3
-02	–	+	–	–	–	–	−4	–	ND	+2
-03	–	–	+	–	–	–	+4	–	ND	+4
-04	–	–	–	–	+	–	+2	–	ND	+2
-05	–	–	–	–	–	+	−3	–	ND	+3
-06	+	+	–	–	–	–	−1	–	ND	+5
-07	+	–	+	–	–	–	+7	–	ND	+1
-08	+	–	–	–	+	–	+5	–	ND	+5
-09	–	+	–	+	–	–	−1	–	ND	+5
-10	–	+	–		–	+	−7	–	ND	+5
-11	–	–	+	+	–	–	+7	–	+	+1
-12	–	–	+	–	–	+	+1	–	+	+1
-13	+	–	–	–	–	+	0	+	ND	0
-14	–	+	+	–	–	–	0	+	ND	0
-15	–	–	–	+	–	+	0	+	ND	0
-16	+	–	–	+	–	–	+6	+	ND	0
-17	–	–	+	–	+	–	+6	+	+	0

[a] Sau I*, variant of *Sau*I site modification that yielded only a 2-base addition.
[b] ND, not done.

of *cis*-acting essential sequences, the generation of DI-H4 RNAs too short to support replication, or some structural requirements of the nucleocapsids preventing any change in the number of nucleotides. These possibilities were made likely by the large size deletions performed. Care was then taken to disrupt as little as possible the RNA sequence in order to learn the rules governing replication. Consequently, 2- to 7-nucleotide deletions or insertions only were produced by cutting and filling in at different unique sites along the sequence. A total of 17 derivatives of pSV-DIH4/Rbz were produced in this way (Table 2, pSV-DIH4/Rbz-01 through -17) and checked in replication assays. Figure 3 presents the Northern blot analysis of the five derivatives (samples 14, 15, 13, 16, and 17) which were successfully and efficiently amplified, and of a selected sample of four derivatives (samples 02, 03, 05, and 01) which were, relative to the previous samples, negative for replication, although a faint signal could sometimes be detected (see in this particular experiment samples 03 and 01). Table 2 summarizes all of the results obtained. The following observations are relevant. (i) The addition of a total of 1, 2, 3, 4, 5, or 7 nucleotides led to negative results, as did the removal of a total of 1, 3, 4, or 7 nucleotides, regardless of the site of modification (Table 2, pSV-DIH4/Rbz and pSV-DIH4/Rbz-01 through -12). (ii) The addition of 3 or 4 nucleotides, with the compensatory removal of 3 or 4 nucleotides at different sites, reestablished the high replication ability (pSV-DIH4/Rbz-13 through -15). (iii) The addition of a total of 6 nucleotides by stepwise additions of 3 plus 3 or 4 plus 2 nucleotides at different locations led to efficient replication (pSV-DIH4/Rbz-16 and -17). (iv) The resulting number of nucleotide changes and not the position of the changes appeared to be important. For instance, a 3-base addition at the *Cel*II site abolished replication (sample pSV-DIH4/Rbz-01). The same modification, when corrected by a 3-base removal at the *Dra*III site (sample pSV-DIH4/Rbz-13) or by a 3-base addition at the *Sau*I site (sample pSV-DIH4/Rbz-16), was no longer negative. As in the case of large internal deletions, the negative results seen here could not be explained by a lack of T7 RNA transcript synthesis nor by a lack of ribozyme activity (not shown).

Encapsidation of nonreplicated DI-H4 RNAs. For replication to take place, a proper template recognized by the viral polymerase has to first be formed. This step involves encapsidation by the NP protein of the T7 RNA transcripts. Encapsidation must, of course, have taken place for the DI RNAs to have successfully replicated. Whether this was the case for the derivatives that did not replicate was open to question. Two replicating and two nonreplicating DI-H4 RNAs were therefore compared for their abilities to be encapsidated when synthesized in the presence of NP alone (Fig. 4). Figure 4A illustrates the replication abilities of the RNAs used. In the presence of the three SV functions but not in the presence of NP alone, DI-H4 RNA and the derivative pSV-DIH4/Rbz-17 efficiently replicated, while derivatives pSV-DIH4/Rbz-12 and -11 did not. However, examination of the T7 RNA transcripts (with a probe of negative polarity [Fig. 4B]) showed that in the presence of NP alone (or in conjunction with P/C and L) these transcripts were all found in nucleocapsid structures (banding in CsCl gradients). Note that the RNAs recovered from the nucleocapsids in the absence of replication were slightly smaller than those seen in the presence of replication (for the most obvious example, compare lanes 3 and 12 with lanes 5 and 14 in Fig. 4B). This is likely to reflect the presence on the agarose gel of only the plus-sense transcript in the absence of

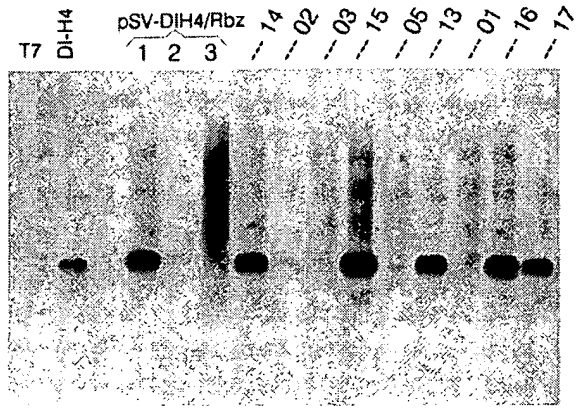


FIG. 3. Replication of DI-H4 RNAs with limited internal modifications. Northern blot analysis of nucleocapsid-purified RNAs from cells transfected with the plasmids expressing the DI-H4 RNA and their derivatives (for a description, see Table 2). For all of the rescue experiments (lanes 1 and 14 through 17), pGem-NP, -P/C, and -L were cotransfected with the plasmid expressing the DI-H4 RNA, except for two samples using pSV-DIH4/Rbz from which the three functions were omitted (lane 2) or in which only pGem-NP was used (lane 3). In vitro T7 RNA transcript synthesized from pSV-DIH4/A linearized with *Bam*HI (lane T7) and nucleocapsid RNA isolated from cells infected with SV DI-H4 (lane DI-H4) are also shown. A 5′ ex riboprobe of positive polarity has been used.

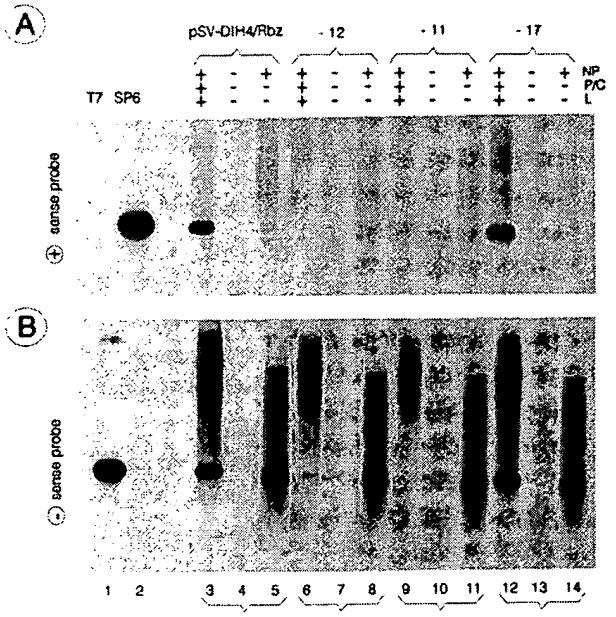


FIG. 4. Encapsidation of DI-H4 RNAs and their derivatives. pSV-DIH4/Rbz and its derivatives pSV-DIH4/Rbz-12, -11, and -17 (see Table 2 for a description) were transfected into CV1 cells, with or without the plasmids expressing the SV replicating functions (NP, P/C, and L) as indicated, in replication or encapsidation assays (see Materials and Methods). Viral nucleocapsids were isolated and the presence of the DI-H4 RNAs was monitored by Northern blot analysis. Lanes: T7 and SP6, in vitro RNA transcripts synthesized from pSV-DIH4/A with T7 and SP6 RNA polymerases, respectively. 5′ ex riboprobes of negative or positive polarity have been used as indicated.

replication, in contrast to the presence of the plus- and minus-sense RNAs when replication takes place. Also note in Fig. 4B the higher-molecular-weight molecules reacting with the minus-strand probe. Most of these molecules were found to be resistant to ribonuclease digestion (data not shown). They are likely to represent mostly encapsidated large T7 RNA transcripts, since they were purified as nucleocapsids. They obviously did not replicate, as evidenced by the absence of their negative-strand counterparts in Fig. 4A, in which residual plasmid contamination is probably responsible for the faint signals observed above the DI RNA bands (Fig. 1, lanes 1 to 5).

DI-H4 RNA replicated from plasmid is infectious. To verify that the nucleocapsids containing DI-H4 RNAs originating from plasmids represented fully competent viral nucleocapsids, a method to produce competent viral stocks containing these nucleocapsids was developed (presented in Fig. 5A). The multiple steps involved were required to provide the nucleocapsids with a viral envelope (adding nondefective virus in step 2) and to produce infectious virus (passage into embryonated eggs in step 3). In step 5, nondefective virus was added to ensure an efficient amplication of the putative infectious DI virus produced in eggs. According to this scheme, DI-H4 RNAs should only be amplified in step 5 when efficiently replicated in step 1. A control in this experiment was, therefore, to omit the three replicating functions in step 1. Figure 5B shows that indeed DI-H4 RNAs were amplified in step 5 (samples $H4^+$ and 17^+). In contrast, they were not detected when replication was not made possible in step 1 (samples $H4^-$ and 17^-). This demonstrated that the AFs contained infectious viral particles with DI-H4 RNAs originating from plasmids. In these experiments, two types of DI-H4 RNAs were used: (i) the original DI-H4 RNA, synthesized from pSV-DIH4/Rbz, whose sequence aligns with that of the nondefective RNA, except for the inverted repeats, and (ii) that produced by pSV-DIH4/Rbz-17, in which 4 and 2 nucleotides were added, at the *Mun*I and *Sau*I sites, respectively, thereby destroying these sites (Table 2). To confirm that the DI RNAs that replicated in step 5 originated from plasmids, a region covering the two *Mun*I and *Sau*I sites was amplified by reverse PCR (see Fig. 6A). The PCR products were then assayed for sensitivity to *Mun*I and *Sau*I digestions. Figure 6B shows the successful amplification of the expected DNA fragment from the four samples presented in Fig. 5B. Not surprising was the recovery of the PCR product from samples $H4^-$ and 17^-, in which DI-H4 did not replicate, since nondefective RNA could also serve as template for the reaction. Note, however, that these PCR products could not be found in the absence of the reverse transcription reaction (RTase − lanes in Fig. 6B), excluding the fact that the plasmids used in the original transfections (step 1 in Fig. 5A) could have been carried along. Digestions with *Mun*I and *Sau*I are presented in Fig. 6C. In the control samples, the 371-nucleotide PCR products originated from pSV-DIH4/Rbz-17 showed total resistance (sample p17), in contrast to sample pH4, which was made from plasmid pSV-DIH4/Rbz. Of the four samples produced from the RNAs presented in Fig. 5 (samples $H4^+$, $H4^-$, 17^+, and 17^-), only the one containing DI-RNA-17 (17^+) led to a PCR product resistant to both restriction enzymes. All of the samples contained, on the other hand, a 371-base fragment sensitive to digestion, since they all contained nondefective viral RNA which served equally as a template for the reverse PCRs. This experiment unequivocally demonstrated that the RNAs orig-

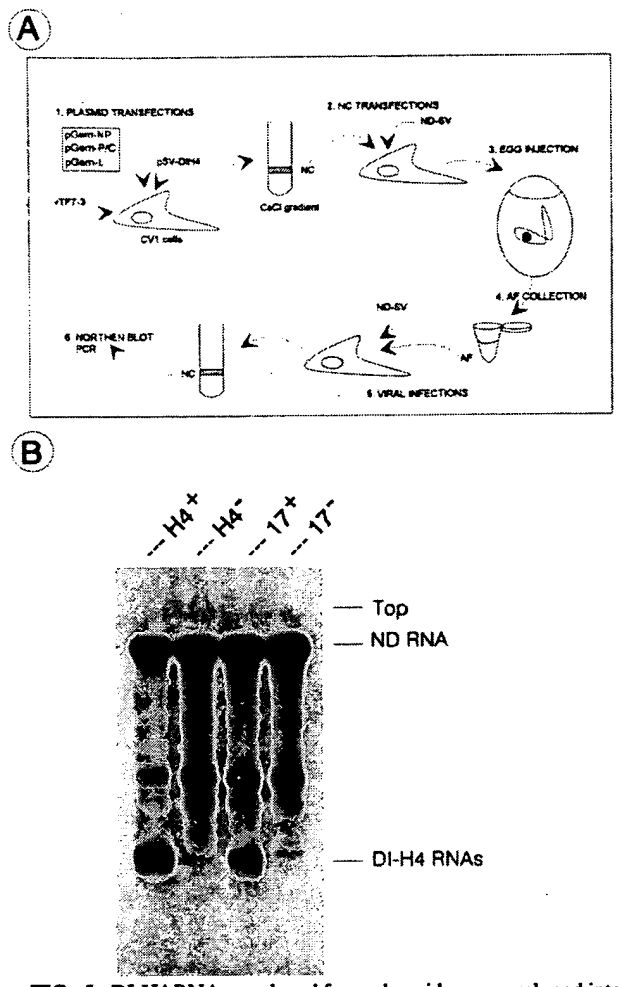


FIG. 5. DI-H4 RNAs produced from plasmids are enveloped into competent virus particles. (A) Scheme of the steps leading to infectious virus from plasmid transfection (for more details, see Materials and Methods). In step 2, the nucleocapsids (NC) isolated from step 1 were transfected and infected with nondefective (ND) SV. In step 3, the whole cells were injected into the embryonated eggs. In step 5, AF collected from the eggs was mixed with nondefective SV. In step 6, the PCR analysis presented in Fig. 6 is shown. (B) Northern blot analysis of the nucleocapsid RNAs amplified in step 5. H4 or 17 samples in their respective lanes are nucleocapsid RNAs isolated from assays with, in the original transfection (step 1), respectively, pSV-DIH4/Rbz and pSV-DIH4/Rbz-17, with or without the three replicating functions (samples $H4^+$ and 17^+ and $H4^-$ and 17^-). The whole procedure was carried out in parallel for each of the four samples. ND RNA, nondefective RNA.

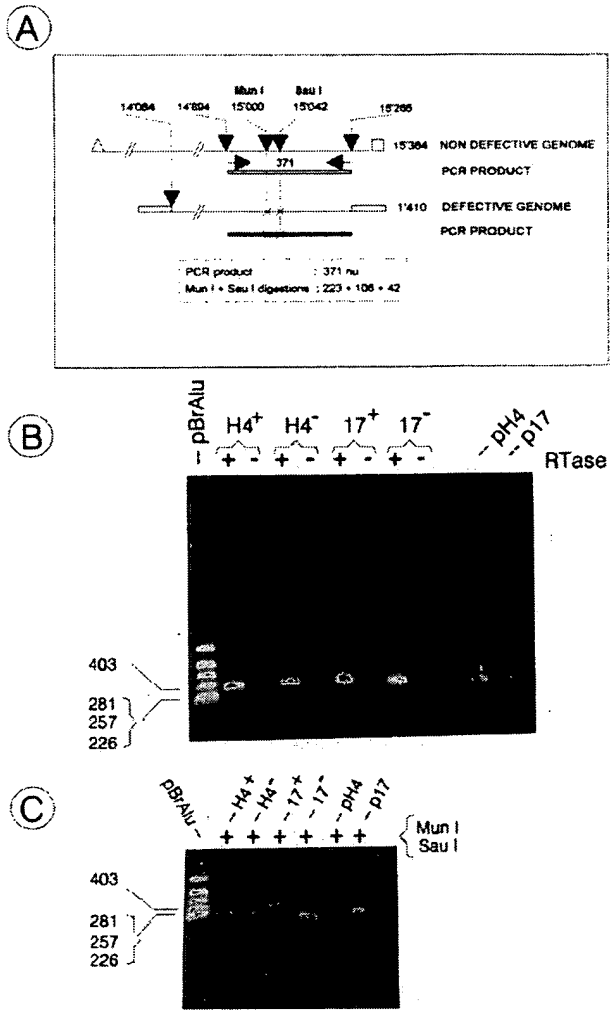


FIG. 6. Reverse PCR analysis of the DI-H4 RNAs originating from plasmids and properly enveloped in competent virus particles. (A) Scheme of the reverse PCR method (for details about primers and reactions, see Materials and Methods). The reverse PCR amplifies a product of 371 nucleotides from both the nondefective and the DI SV RNAs. When amplified from pSV-DIH4/Rbz-17, however, this product is resistant to digestion with *Mun*I and *Sau*I. (B) A 1% agarose gel analysis of the reverse PCR products obtained with nucleocapsid RNAs purified from cells in step 5 as templates (Fig. 5A) after the original transfection (step 1 in Fig. 5A) with either pSV-DIH4/Rbz (samples H4) or pSV-DIH4/Rbz-17 (samples 17) with or without the three replicating functions (samples $H4^+$ and 17^+ and $H4^-$ and 17^-). Lanes pH4 and p17 contain PCR products of reactions with pSV-DIH4/Rbz and pSV-DIH4/Rbz-17 plasmids. Row RTase indicates whether reverse PCRs included the reverse transcriptase. Molecular markers (in bases) are on the left. (C) A 1% agarose gel analysis of the sensitivity of the PCR products to digestion with *Mun*I or *Sau*I. For details, see the legend to panel B.

inating from plasmids were not only efficiently replicated but behaved like bona fide subgenomic viral RNAs in that they were replicated by the helper virus and enveloped in competent virus particles.

Total length of the DI-H4 insert in pSV-DIH4/Rbz. When DI-H4 RNA was cloned into pSV-DIH4/A, an estimate of its length based on partial sequencing of crucial regions of the insert and on alignment with published sequences (24, 25) led to a total of 1,411 nucleotides (3). Since then, the complete sequence of the insert has been done (data not shown). A single-base deletion was found at position 111 downstream from the genomic 3′ end (for details, see reference 3). Therefore, the total number of nucleotides in the insert is 1,410.

DISCUSSION

In the experiments presented here, the replication of DI RNAs from plasmids was detected by Northern blotting, a

relatively insensitive method. This argues for a high efficiency of replication, at best 1 order and at worst 2 orders of magnitude lower than that seen in natural virus infections. This estimate does not take into account the fraction of the cells transfected. The replication mechanisms were, therefore, likely to be similar to those seen in natural infections. The absence of replication in our experiments, however, should not be interpreted as absolute. Clearly, low-efficiency replication leading to amounts of RNA lower by 2 or 3 orders of magnitude would have been scored negative in our assays (see Results and text below). The beneficial effect of the addition of the self-cleaving activity which generates the correct T7 RNA 3' end has been recognized before (20) and is likely to result from a higher transfection efficiency as well as from a greater stability of the transfected plasmids. The consistency of this self-cleaving activity (measured in vitro for all of the constructions used here) excludes its implication in the cases in which failure to replicate was observed.

Starting from a pSV-DIH4/Rbz plasmid that led to efficient DI-H4 RNA replication, large internal deletions in the DI-H4 RNA coding sequence abolished replication. This was unexpected, since the end sequences, generally accepted to be critical for replication for SV as well as for other members of the negative-stranded RNA virus family (4, 6, 15, 17), were perfectly conserved. Because of the large size deletions, it was, however, difficult to easily explain these failures. The removal of essential *cis* sequences, the generation of too-short RNAs, and the modification of a critical RNA length required to provide specific NP-RNA interactions were considered. Among these possibilities, the last was judged most easily open to experimentation and was therefore tested. Limited nucleotide additions or deletions leading to unsuccessful or successful replication were indeed indicative of the mechanisms involved. Disruption of the correct sequence at various positions was found to be irrelevant to efficient replication as long as the total number of nucleotides remained unchanged (Table 2, pSV-DIH4/Rbz-13 to -15). Moreover, in two cases, two-step additions of a total of 6 nucleotides in two different sites led to successful replication, while the modification of each of these individual four sites led to failures (Table 2, pSV-DIH4/Rbz-16 and -17). Therefore, if the total number of nucleotides appeared critical, the addition of 6 nucleotides was allowed. The simplest interpretation of these results is that the total number of nucleotides must be a multiple of six. This rule (rule of six) is verified in our experiments (Table 2, last column) and is also in agreement with the total number of nucleotides of the nondefective genome and of the DI-H4 genome produced from pSV-DIH4/Rbz, which amount to 15,384 (24, 25) and 1,410, respectively. This rule provides a likely explanation for the failure to replicate the large internal deletions as well, since the DI-H4 RNAs were shortened by 285, 782, and 1,114 nucleotides; none of these numbers is a multiple of six. Other causes for failure are possible.

In the failures to replicate, encapsidation of the T7 transcripts (starting at the 5' end) did not appear to be the limiting step, since the transcripts were found in nucleocapsid structures banding on CsCl gradients (Fig. 4). Assuming, therefore, that correct encapsidation of the T7 transcripts takes place anyway, the critical step for allowing replication may become the correct encapsidation of the 3' extremity. This extremity is crucial in that it represents the promoter for the synthesis of the strand complementary to the T7 RNA transcript. The 5' end contains the signal for proper positioning of the first NP subunit and for encapsidation initiation this signal is likely to be not only geographic (the 5' extremity) but also a specific nucleotide sequence (shown for vesicular stomatitis virus [VSV] to lie among the first 14 nucleotides [1, 2]). Subsequent NPs would then be sequentially positioned by contacting the previous NP and establishing specific contacts with a series of nucleotides. The data presented here support evidence that the number of nucleotides that NP contacts is six. Therefore, from 6 to the next 6 nucleotides, the NP subunits would be sequentially added until they reach the 3' RNA extremity. There, if the RNA contains a number of nucleotides which is a multiple of six, the last 6 nucleotides will be correctly covered by the last NP. In contrast, extra nucleotides would lead either to dangling nucleotides or to an NP subunit making contact with fewer than 6 nucleotides. Our data suggest that neither structure can serve as a proper replication promoter. In this model, the NP proteins have to be tightly packed on the RNA (full resistance to nuclease has been previously shown, regardless of the extension of the helical pitch [11]) and not free to move along the RNA.

That each NP molecule specifically contacts 6 nucleotides is in agreement with the total number of NP proteins per nondefective nucleocapsid estimated by analysis of electron microscopic pictures (7, 8). Egelman and colleagues in particular come up with a stoichiometry of 6 nucleotides to 1 NP subunit on the basis of their observations (13 NP subunits per helical turn, 210 helical turns per nucleocapsid) and on the rationalization that nonbroken helical symmetry suggests an integer stoichiometry between bases and NP subunits. With the total number of NP subunits that they calculate ($13 \times 210 = 2{,}730$), the deduced stoichiometry becomes 5.6:1 (15,384/2,730), a noninteger stoichiometry. Egelman and colleagues favor a 6:1 stoichiometry, since this would not break the helical symmetry and would fit with the estimation of the total number of NP subunits better than a 5:1 stoichiometry, requiring more than 3,000 NP subunits. It is certainly comforting that structural data are in agreement with functional assays.

In a recent paper, Pattnaik and colleagues (20) have reported, using a system similar to the one used here, efficient encapsidation and replication of a VSV DI RNA expressed from plasmid. They made the interesting observation that 4 to 5 extra nucleotides at the 5'-end T7 transcript were tolerated, in that they did not kill replication. They were, however, trimmed in the DI RNA that was successfully amplified. On the other hand, as few as 2 extra nucleotides at the 3' end of the T7 transcript abolished replication. In view of the results presented here, and assuming that a basic rule such as the rule of six applies to VSV, these results can be interpreted as follows. Correct positioning of the first NP only takes place internally at the true viral RNA 5' end by recognition of a specific nucleotide sequence (leaving dangling extra nucleotides). Encapsidation would then proceed to the 3' end, regardless of the nucleotide sequence, but with the correct integer NP/nucleotide stoichiometry observed. At the 3' end, only the precise number of nucleotides would be tolerated to generate the replication promoter for the complementary strand (no extra nucleotides allowed). That the synthesis of the complementary strand leads to a successful template for the next round of replication, despite the dangling 5' extra nucleotides, could be explained if the unprotected 5' nucleotides are cleaved. Alternatively, they could not be copied by the viral replicase, because they are not covered by NP. That correction at the 3' end does not take place (no trimming) may suggest that extra nucleotides are not dangling but rather are covered with an extra NP protein, enough to be protected,

but not properly so that a correct replication promoter is formed.

The rule of six does not preclude the need for a particular nucleotide sequence at the RNA ends. As suggested before and mentioned above, these 11 to 14 nucleotides conserved between the plus and minus strands are likely to constitute, when in the proper phase with the NP proteins, a specific signal required for forming an adequate template for replication. Moreover, the rule of six does not exclude other possible requirements, such as total minimal RNA length or particular *cis* RNA sequences. Note that an RNA construct containing the CAT gene sequence flanked by the SV 3'- and 5'-end sequences has been successfully replicated with nondefective SV to provide the helper functions (19). The total number of nucleotides in this RNA amounts to 924, a multiple of six. However, the replication of this RNA lacking all but the 145 nucleotides at the 3'-end and 119 nucleotides at the 5'-end SV sequences was only detected by measurement of the CAT activity (a highly sensitive assay compared with Northern blotting). This could suggest the need for internal sequences, absent from this synthetic construct, to reach higher replication efficiency. Alternatively, this could reflect the difference in plus- and minus-strand replication promoters. In copy-back DI RNAs, the promoter responsible for minus-strand synthesis (generally accepted to be of high efficiency) is present on both RNA strands (contained in the inverted repeat). This is obviously not the case for the RNA synthetic construct (analogous to an internal deletion DI RNA), which has retained the ends of the genomic RNA. Note that when a synthetic RNA construct of the type reported by Park et al. (19) was confronted with the rule of six, the correct number of nucleotides had a positive effect (three- to fivefold) on the efficiency of replication. This, however, was still marginal (i.e., only detected after PCR amplification) compared with the level of replication reached with the natural copy-back DI RNA (11a).

It is therefore possible that the rule of six shows a significant effect on replication only when all of the other rules needed for replication are observed. The rule of six would thus become necessary for efficient replication in conjunction with other rules needed for replication. This interpretation could account for the out-of-phase derivatives exhibiting sporadic replication abilities at the limit of detection (Fig. 3, samples 03 and 01) or for a marginal effect on synthetic constructs obeying the rule of six (cf. constructs in reference 11a).

Successful replication by negative-stranded RNA virus or virus functions of RNAs produced from plasmids has been reported for other viruses as well. Apart from the VSV DI RNA and the SV CAT construct already discussed above, RNA molecules containing the reporter gene CAT sequence flanked by 3' and 5' viral RNA end sequences of influenza A or respiratory syncytial viruses have been replicated by their respective homologous viruses (4, 16). These artificial defective RNAs were correctly enveloped in virus particles. Among these different RNAs, only the VSV DI RNA replicated with an efficiency comparable to that observed here (direct detection of the DI RNA), therefore potentially fulfilling the conditions for the rule of six to apply. However, although the natural VSV DI RNA is 2,208 (a multiple of six) nucleotides long, the DI RNA which was reported to efficiently replicate from plasmid is 1 nucleotide longer (20). Moreover, Wertz and colleagues recently reported the successful replication of a series of deletions of their original DI RNA for which a strict correlation with the rule of six was not observed (26). Among the CAT constructs, for which the rule of six may not apply with full potential according to our interpretation (see above), the influenza constructs of Luytjes et al. (16) fitted the rule. This, however, has to be balanced by the fact that for influenza virus, none of the natural full-length genes are made up of nucleotides in multiples of six (see reference 13 for a review).

In conclusion, the available sequence data do not, at present, speak for a wide application of the rule of six. It is not clear whether a more extended sequence comparison will bring more information, since for most of the reported sequences, the degree of confidence does not lie within plus or minus 1 or 2 nucleotides. Rather than elucubrate the general prevalence of the rule of six, one would like to propose that although the number of nucleotides per nucleocapsid protein unit may depend on the type of virus (on the size of the nucleocapsid protein), the rule that the stoichiometry of nucleotide per NP protein must be an integer has to be considered for all of the viruses containing a nucleocapsid with tight helical symmetry.

ACKNOWLEDGMENTS

We are indebted to Tatsuo Shioda, who made us aware of the possibility of the rule of six. We also thank Geneviève Mottet for excellent technical assistance and Joseph Curran and Daniel Kolakofsky for fruitful interactions of all sorts.

This work was supported by a grant from the Swiss National Foundation for Scientific Research and grants from Sandoz, Ciba-Giegy, and the Société Académique de Genève to L.R. P.C. is a recipient of fellowships from the Schweizerische Akademie der Medizinischen Wissenschaften, the Holderbank-Stiftung, the Jangen-Pöhn-Stiftung, and the Roche and the de Reuter Foundations.

REFERENCES

1. **Blumberg, B. M., C. Giorgi, and D. Kolakofsky.** 1983. N protein of vesicular stomatitis virus selectively encapsidates leader RNA in vitro. Cell 32:559–567.
2. **Blumberg, B. M., and D. Kolakofsky.** 1981. Intracellular vesicular stomatitis virus leader RNAs are found in nucleocapsid structures. J. Virol. 40:568–576.
3. **Calain, P., J. Curran, D. Kolakofsky, and L. Roux.** 1992. Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA. Virology 191:62–71.
4. **Collins, P. L., M. A. Mink, and D. S. Stec.** 1991. Rescue of synthetic analogs of respiratory syncytial genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. Proc. Natl. Acad. Sci. USA 88:9663–9667.
5. **Curran, J., R. Boeck, and D. Kolakofsky.** 1991. The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. EMBO J. 10:3079–3085.
6. **Curran, J. A., and D. Kolakofsky.** 1991. Rescue of a Sendai virus DI genome by other parainfluenza viruses: implications for genome replication. Virology 182:168–176.
7. **Egelman, E. H., S.-S. Wu, M. Amrein, A. Portner, and G. Murti.** 1989. The Sendai virus nucleocapsid exists in at least four different helical states. J. Virol. 63:2233–2243.
8. **Finch, J. T., and A. J. Gibbs.** 1970. Observations on the structure of the nucleocapsids of some paramyxoviruses. J. Gen. Virol. 6:141–150.
9. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA 83:8122–8126.
10. **Galinsky, M. S., and S. L. Wechsler.** 1991. The molecular biology of the paramyxovirus genus, p. 41–82. *In* D. W. Kingsbury (ed.), The paramyxoviruses. Plenum Press, New York.
11. **Heggeness, M. H., A. Scheid, and P. W. Choppin.** 1981. The relationship of conformational changes in the Sendai virus

nucleocapsid to proteolytic cleavage of the NP polypeptide. Virology 114:555–562.

11a. **Jacques, J.-P., and D. Kolakofsky.** Personal communication.

12. **Kast, W. M., L. Roux, J. Curran, H. J. J. Blom, A. C. Voordouw, R. H. Meloen, D. Kolakofsky, and C. J. M. Melief.** 1991. Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with a free synthetic peptide. Proc. Natl. Acad. Sci. USA 88:2283–2287.

13. **Lamb, R. A., and P. W. Choppin.** 1983. The gene structure and replication of influenza virus. Annu. Rev. Biochem. 52:467–506.

14. **Lamb, R. A., B. W. J. Mahy, and P. W. Choppin.** 1976. The synthesis of Sendai virus polypeptides in infected cells. Virology 69:116–131.

15. **Li, X. Q., and P. Palese.** 1992. Mutational analysis of the promoter required for influenza virus virion RNA synthesis. J. Virol. 66:4331–4338.

16. **Luytjes, W., M. Krystal, M. Enami, J. D. Parvin, and P. Palese.** 1989. Amplification, expression, and packaging of a foreign gene by influenza virus. Cell 59:1107–1113.

17. **Mink, M. A., D. S. Stec, and P. L. Collins.** 1991. Nucleotide sequences of the 3′ leader and 5′ trailer regions of human respiratory syncytial virus genomic RNA. Virology 185:615–624.

18. **Mottet, G., and L. Roux.** 1989. Budding efficiency of Sendai virus nucleocapsids: influence of size and ends of the RNA. Virus Res. 14:175–188.

19. **Park, K. H., T. Huang, F. F. Correia, and M. Krystal.** 1991. Rescue of a foreign gene by Sendai virus. Proc. Natl. Acad. Sci. USA 88:5537–5541.

20. **Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell 69:1011–1020.

21. **Perrotta, A. T., and M. D. Been.** 1990. The self-cleaving domain from the genomic RNA of hepatitis delta virus: sequence requirements and the effects of denaturants. Nucleic Acids Res. 18:6821–6827.

22. **Perrotta, A. T., and M. D. Been.** 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature (London) 350:434–436.

23. **Roux, L., and J. J. Holland.** 1979. Role of defective interfering particles of Sendai virus in persistent infections. Virology 93:91–103.

24. **Shioda, T., Y. Hidaka, T. Kanda, H. Shibuta, A. Nomoto, and K. Iwasaki.** 1983. Sequence of 3,687 nucleotides from the 3′ end of Sendai virus genome RNA and the predicted amino acid sequences of viral NP, P and C proteins. Nucleic Acids Res. 11:7317–7330.

25. **Shioda, T., K. Iwasaki, and H. Shibuta.** 1986. Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN, and L proteins. Nucleic Acids Res. 14:1545–1563.

26. **Wertz, G. W. et al.** 1993. Presented at the Reverse Genetics of Negative-Stranded RNA Viruses Meeting, Madrid, February 1993.

27. **Wu, H.-N., Y.-J. Lin, F.-P. Lin, S. Makino, M.-F. Chang, and M. M. C. Lai.** 1989. Human hepatitis delta virus RNA subfragments contain an autocleavable activity. Proc. Natl. Acad. Sci. USA 86:1831–1835.

VIROLOGY 191, 62–71 (1992)

Molecular Cloning of Natural Paramyxovirus Copy-Back Defective Interfering RNAs and Their Expression from DNA

PHILIPPE CALAIN, JOE CURRAN, DANIEL KOLAKOFSKY, AND LAURENT ROUX[1]

Department of Genetics and Microbiology, University of Geneva Medical School, C.M.U., 9 Avenue de Champel, 1211 Geneva 4, Switzerland

Received December 13, 1991; accepted July 13, 1992

Using the unique sequence organization of copy-back defective interfering (DI) RNAs of paramyxoviruses, Sendai virus (SV), and measles virus copy-back DI RNAs were PCR amplified and cloned, without having to separate them from their helper nondefective genomes. The cloning was designed so that T7 polymerase transcription of the plasmids would generate DI RNAs with the exact 5′ and 3′ ends. The SV DI clone, transcribed from the plasmid in BHK cells using T7 polymerase produced by a vaccinia virus recombinant, was encapsidated and replicated by the SV-L, P/C, and NP proteins expressed from cloned genes. Such experiments open the possibility of examining the *cis*-acting sequences involved in viral multiplication directly, without using indirect markers such as CAT activity. © 1992 Academic Press, Inc.

INTRODUCTION

Defective interfering (DI) viruses contain deleted genomes, which spontaneously arise during infections due to errors of the replicase complex. Once generated, they are amplified preferentially over their helper nondefective or standard (St) genome, with which they also interfere in the process of replication (for reviews, see Perrault, 1981; Schlesinger, 1987). The presence of DI viruses in coinfections generally results in the attenuation of the cythopathic effects of the St virus, thereby favoring the establishment of persistent infections (reviewed by Holland *et al.*, 1980). In cell cultures, DI viruses drive rapid virus evolution because there is now pressure for the nondefective virus to escape interference (reviewed by Holland *et al.*, 1982). DI viruses have also been shown to protect animals from lethal infections or to modulate the course of the infections by lowering infectious virus replication, inducing interferon, or modulating the immune system (reviewed in Holland, 1987; Huang, 1987; Barrett and Dimmock, 1986; Roux *et al.*, 1991).

The most frequently described DI genomes of nonsegmented (−)RNA viruses, such as rhabdoviruses and paramyxoviruses, are of the "copy-back" type. These are so called because they can be generated during the copying of the antigenome by the replicase leaving its template and resuming synthesis on the nascent genome chain it is still carrying. The replicase then finishes the chain by "copying back" to the beginning of the nascent genome chain at the same end of the genome map whence it started. The resulting DI genome is then deleted of all sequences downstream of the crossover point (on the antigenome template), and has added at its 3′ end the complement of its own 5′ end. The complementary ends of these chains, which are 50–200 nucleotides (nt) long (Perrault, 1981), are responsible for the circular "panhandle" structures these genomes form as naked RNA. Sequence information is available for some vesicular stomatitis virus (VSV) and Sendai virus (SV) DI species (Perrault, 1981; Re, 1991), and one sequence of a measles virus (MV) DI has also been reported (Enami *et al.*, 1989).

Many questions remain about how DI viruses attenuate St virus infections and favor the establishment of persistent infections. Their participation in virus infections in nature is also as yet poorly documented, although results obtained in experimental animal systems (Barrett and Dimmock, 1986; Huang, 1987) suggest their potential importance (Roux *et al.*, 1991). To help examine these questions, it would of course be useful to be able to simply clone natural DI genomes as DNA. DNA clones could then hopefully be used to produce variant genomes carrying targeted mutations.

The molecular cloning of copy-back DI RNA is not straightforward, due to the presence of the long inverted terminal repeats. We describe here a series of experiments, based on polymerase chain amplification (PCR), which allowed us to detect and clone as plasmids paramyxoviruses copy-back DI genomes, in the presence of the St genome. A method is also presented which allows efficient synthesis of DI RNA from DNA where cloning into plasmids would not be successful. The plasmid DNAs were then used to make precise copies of the DI genome as RNA, and these were shown to be functional by their ability to be encapsidated and replicated by the viral NP, P, and L proteins, also expressed from plasmid DNAs.

[1] To whom reprint requests should be addressed.

0042-6822/92 $5.00

MATERIALS AND METHODS

Viruses and cells

MV DI P6 stock, generated after six undiluted passages in Vero cells has been described before (Calain and Roux, 1988). It contains a major subgenomic RNA of about 1000 bases estimated by Northern blot analysis. Sendai virus (SV) DI-H4 stock obtained after four undiluted passages in embryonated eggs contains a 14 S subgenomic RNA (Leppert *et al.*, 1977). Both DI RNAs were defined as copy-back by their ability to react only with 5′ end genomic probes. DI-H4 RNA was further shown to contain a panhandle of 110 nucleotides (Leppert *et al.*, 1977). Vero and baby hamster kidney cells, grown in regular MEM supplemented with 5% fetal calf serum, were used for production of MV and SV viral nucleocapsid RNAs, respectively.

Purification of nucleocapsid RNAs

Cells infected with MV P6 or SV DI-H4 (m.o.i. of 1) were harvested, respectively, at 48 or 24 hr postinfection. Intracellular nucleocapsids were isolated on cesium chloride gradients according to a previously published method (Kolakofsky, 1976). RNA was recovered from the nucleocapsid pellet by extraction with phenol/chloroform and by ethanol precipitation in presence of t-RNA carrier.

Polymerase chain reaction

Reverse transcription was carried with MMLV reverse transcriptase (BRL) according to the manufacturer's protocol, and using 20 pmol of oligonucleotide primer in a 50-μl reaction. Typically, the amount of nucleocapsid RNA isolated from 10^6 infected cells was reverse transcribed. One-fiftieth of the reverse transcriptase reaction was further used in 100-μl PCR reactions.

Taq polymerase (Biofinex) or Amplitaq (Cetus) were generally used at a concentration of 2 units/100 μl reactions in 67 m*M* Tris–HCl, pH 8.8, 16.6 m*M* ammonium sulfate, 10 m*M* β-mercaptoethanol, 170 μg/ml bovine serum albumin, 3 m*M* $MgCl_2$, 50 μM of each d-NTP, and 20 pmol of each amplimer. Alternatively, when 10% DMSO was used, the buffer was modified to contain 80 μg/ml bovine serum albumin, 6.7 μM EDTA, 6.7 μM $MgCl_2$, and 1 m*M* of each d-NTP (Kogan *et al.*, 1987).

Stoffel fragment of Taq polymerase (Cetus) is described under Results. Specific information about the nature and the number of cycles is also given under Results.

Oligonucleotides

The various oligonucleotides used in reverse transcription and PCR amplification are presented in Table 1.

Plasmids and bacteria

Escherichia coli Epicurian Sure cells (Stratagene) were used for transformations. When the PCR amplification products did not contain specific cloning sites, they were phosphorylated, blunt ended with Klenow, and cloned in *Sma*I-digested, dephosphorylated pGEM4. Recombinant clones were screened by the method of Grunstein and Hogness (Grunstein and Hogness, 1975) using probes representing the 5′ end genomic sequences [pMV5deltaB (Calain and Roux, 1988) and 5′ ex (Mottet and Roux, 1990) probes, respectively, for MV and SV]. The PCR amplification products containing the T7 polymerase promoter and flanked by appropriate cloning sites (see Results) were digested and cloned into the appropriately linearized and dephosphorylated pSP65 so that the cloned T7 polymerase promoter faced the SP6 polymerase promoter.

Sequencing

Sequences were established with the T7 Sequencing Kit (Pharmacia) using as template the purified plasmids. For sequencing purposes, additional primers to those described in Table 1 were used encompassing nucleotides 14,176–14,196 for SV, and nucleotides 15,651–15,671, 15,615–15,594, 15,272–15,251, 15,143–15,122 for MV (for numbering convention, see legend of Table 1, *a*). Moreover, an oligonucleotide covering nucleotides 2941–2960 of pSP65 was used to obtain the border sequences of the cloned inserts.

In vitro transcription

In vitro transcriptions were performed using T7 polymerase (Biofinex, Switzerland, or Biolabs) following the conditions provided by the manufacturers. Copy-back transcript polarity was of course according to the polarity of the sequence located between the inverted repeats.

RESULTS

Because of their unusual sequence organization, one can design PCR amplifications of DI RNAs which cannot occur on St genomes. The simplest of these approaches is to use only a single amplimer complementary to the 3′ end of the St antigenome, which is also complementary to both 3′ ends of the DI "genomes" and "antigenomes." The copy-back DIs are,

TABLE 1

OLIGONUCLEOTIDES

	Sense	Position[a] (5′–3′)	Sequence (5′–3′)
SV PCR primers			
SVA2[b]	(−)	15,384–15,356[c]	ATGTCGACTAATACGACTCACTATA**GCCAGACAAGAGTTTAAGAGATATGTATC**
SVB2[d]	(−)	15,384–15,356[c]	ATTGGATCCGAATGCTA**CCAGACAAGAGTTTAAGAGATATGTATC**
SVA1	(−)	14,917–14,894	**AGTCTTCTAGAAATTGTCTTCAAC**
SVB1	(+)	14,894–14,917	**GTTGAAGACAATTTCTAGAAGACT**
MV PCR primers			
MVA2[b]	(−)	15,893–15,865[c]	AGGTCGACTAATACGACTCACTATAA**CCAGACAAAGCTGGGAATAGAAACTTCG**
MVB2[d]	(−)	15,893–15,865[c]	ATTGGATCCGAATGCTA**CCAGACAAAGCTGGGAATAGAAACTTCG**
MVA1	(−)	15,638–15,616	**TCTGGTGTAATCTAGTATCAGA**
MVB1	(+)	15,107–15,127	**ATGACAGATCTCAAGGCTAAC**
MVRT	(−)	15,884–15,865	**AGCTGGGAATAGAAACTTCG**
Overhangs[e]			
T7*Sal*	—	—	AGGTCGACTAATACGA
BamBsm	—	—	ATTGGATCCGAATGCT

[a] Position in the viral genome. SV and MV genomes are respectively 15,384 and 15,893 nucleotides long, with the 3′ end nucleotide being number 1.

[b] SVA2 and MVA2 contain *Sal*I and T7 promoter sequences depicted in regular characters at their 5′ ends.

[c] Numbers refer to viral sequences depicted in bold characters.

[d] SVB2 and MVB2 contain at their 5′ ends *Bam*HI and *Bsm*I sequences depicted in regular characters.

[e] Primers which allow extension from the nonviral sequences of MVA2, SVA2, MVB2, and SVB2.

of course, chimeras in which each chain contains both genomic or (−) and antigenomic or (+) sequences. However, for ease of discussion, we will continue to use this terminology, which refers here only to sequences between the terminal repeats. This single amplimer can, at least in theory, amplify copy-back DI RNAs (Fig. 1A). This approach, however, proved unsuccessful. It led at best to amplification of sequences containing only the 3′ end of the (−) copy-back DI RNA. These molecules were portions of these DI RNAs because they contained the chimera junction (divergent sequence point), and probably resulted from rare nonspecific hybridization of the primer to sequences contained between the inverted repeats during the RTase step (data not shown). It appeared that intramolecular hybridization of the terminal repeats must have prevented correct formation of the primer hybrid. One suspects that the length of the inverted repeats must be critical for the successful outcome of this method. A recent report appears to support this, since a vesicular stomatitis virus (VSV) copy-back DI RNA with inverted repeats of only 45 nt has been successfully cloned using a single primer (Pattnaik *et al.*, 1992).

These results, however, suggested an alternative approach in which separate amplification of the two halves of the copy-back was possible. Here, as before, basically a single terminal amplimer is used so that St molecules cannot be amplified. However, two such amplimers are used (A2 and B2 in Fig. 1B) because they contain different nonviral sequences at their 5′ ends (see Table 1), which are useful for subsequent cloning and expression. This method begins with two separate RTase reactions with amplimers A1 and B1 (Fig. 1B), which are complementary and contain an internal restriction site. Here, hybridization of the RTase primers does not have to compete against intramolecular hybridization of the template. The critical step becomes the ability of the RTase to displace the panhandle. Using this approach, the cloning of SV DI-H4 and MV P6-DI RNAs were successful. However, they were carried out with slightly different strategies and are presented separately.

Cloning of the Sendai DI H4 sequence

The Sendai DI-H4 copy-back RNA was characterized previously to be ca. 1200 nt long, with inverted repeats of 110 nt (Leppert *et al.*, 1977). The internal amplimers SVA1 and SVB1 (Table 1) overlap at a unique *Xba*I site (position 14,907, relative to the 3′ end of the St genome; the 5′ end is nt 15,384). The terminal amplimers, SVB2 and SVA2, contained at their 3′ ends 29 nt complementary to the last 29 nt of the 3′ end of the antigenomic RNA. For SVA2, the additional 5′ nonviral sequences contained a *Sal*I site, and a T7 promoter joined to the Sendai sequence so that antigenomic transcripts could be expressed. The first viral nucleotide was also degenerate so that T7 transcripts starting with both A and G at this position can be made. For SVB2, a *Bsm*I site allows for a precise SV 3′ end when the DNA is restricted here, and this is flanked by a *Bam*HI site.

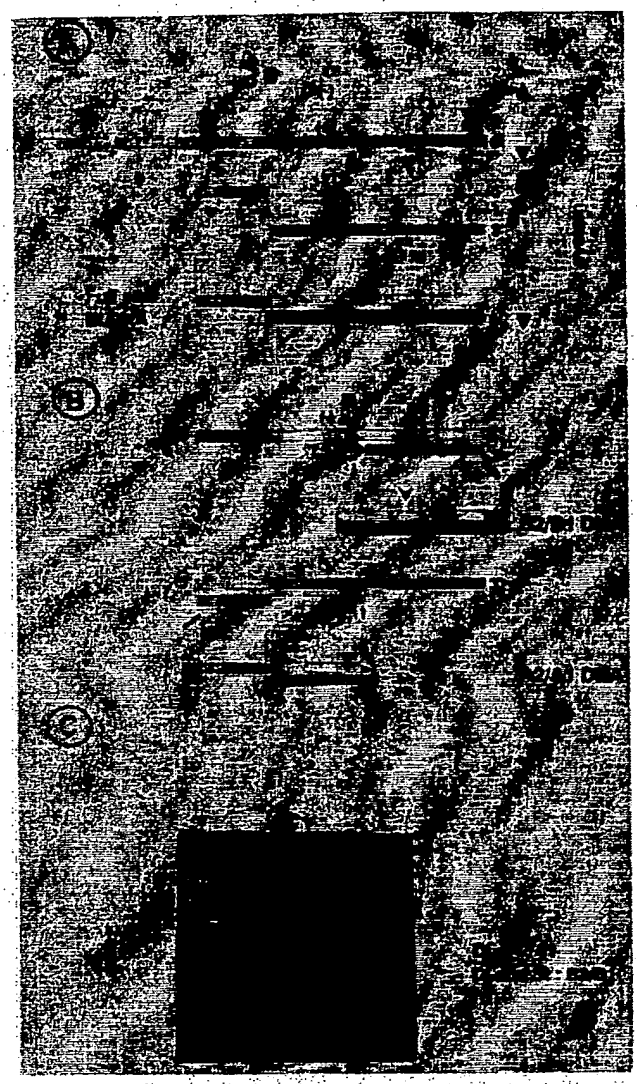


FIG. 1. PCR amplification of copy-back DI RNAs. (A) Amplification of the full-size DI RNA sequence using a unique primer of negative polarity (open arrow) internal to the inverted repeats. The primer serves as RTase primer as well as PCR primers. PCR amplification of the c-DNAs can only take place for the DI RNAs. The polarities of the template molecules are indicated. D, Point of sequence divergence between the St RNA and the DI RNA. (B) Amplification of the DI RNA sequence in two pieces ligated at a unique restriction site, R. c-DNA synthesis, performed in different reactions, are directed by two primers of different polarity, A1 and B1 (see for specific sequences Table 1). In each reaction, c-DNAs complementary to DI as well as St (not shown in the scheme) RNAs are possibly made. Each set of c-DNAs is then amplified with the pair of A2/A1 and B2/B1 primers (for A2- and B2-specific sequences, see Table 1 and Results), leading to the A2/A1 and B2/B1 ds DNA products. B2/B1 product can be derived from St and DI RNAs. A1/A2 product amplification is only possible in the presence of DI RNAs. A1 and B1 can, but need not, overlap at an adequate restriction site, R. (C) PCR amplification of SV DI-H4 RNA. Reverse transcriptase reactions were run as described under Materials and Methods using viral RNA isolated from DI-H4 or St virus infected cells and either SVA1 or SVB1 primers (Table 1). One-hundredth of each reaction was then used for PCR reactions (20 μl) using Amplitaq (Cetus) with the pairs of primers A2/A1 or B2/B1 (denaturation, 96° 10"; hybridization, 50° 10"; elongation, 72° 40"; 30 cycles, Cetus 9600). Ten microliters of the reactions were analyzed on 1% agarose gel electrophoresis. Lane 1, phage lambda DNA digested with *HindIII/EcoRI*. Lane 2, pBr322 DNA digested with *AluI*. Lane 3, SVA1/A2 DNA amplified from DI-H4 RNA. Lane 4, SVB2/B1 DNA amplified from DI-H4 RNA. Lane 5, result of SVA2/A1 amplification from St RNA. Lane 6, SVB2/B1 DNA amplified from St RNA.

Figure 1C shows the results of this approach using both St RNA and a mixture of St and DI RNAs. Fragments of the expected lengths were amplified, and the reactions appeared specific as no products were seen in the A2/A1 reaction when St RNA was the initial template (lane 5). The A2/A1 and B2/B1 fragments were digested with *XbaI/SalI* and *XbaI/Bam*HI, respectively, and cloned in a three-piece ligation into the *Bam*HI/*Sal*I sites of pSP65. Twelve clones were examined, and three carried an insert of about 1.2 kb. The nine others either contained shorter inserts or had lost the *Bam*HI or *Sal*I sites. Partial sequencing of the three candidate clones (Fig. 2B) gave similar sequences, consistent with a copy-back RNA of 1411 nt, with inverted repeats of 110 nt. One clone contained an A at viral position +1 downstream of the T7-promoter (pSV-DIH4/A), and two contained a G at this position (pSV-DIH4/G, Fig. 2C).

Cloning of the MV P6 DI.2 sequence

MV DI-P6 RNA is about 1 kb long and contains the 5′ but not the 3′ end of the St genome (Calain and Roux, 1988). Sequencing of amplification products, obtained with the approach of Fig. 1A showed that two copy-back DI RNAs were present in this stock. Figure 3A shows some of the sequences obtained where two crossover positions at nt 15,136 and 15,079 are evident. Figure 3B outlines the sequences of the two DI RNAs relative to the genomic sequence, and highlights where the polymerase jumped from the antigenome template and resumed synthesis on its own nascent minus strand. Figure 3C shows the predicted primary structure of the two RNAs, P6DI.1 and P6DI.2. They are 893 and 978 nt long, and contain inverted repeats of 135 and 163 nt, respectively. As the two sequences were also obtained using other nested amplimers (not shown), and PCR products were never observed when St RNA was used as the RTase template, both sequences are probably authentic. The P6DI.1 sequence was observed at much lower frequency than that of P6DI.2, and is likely to be a minor species in the P6 DI stock.

To clone these copy-back DI RNAs, a strategy similar to that used for SV was employed, except that the

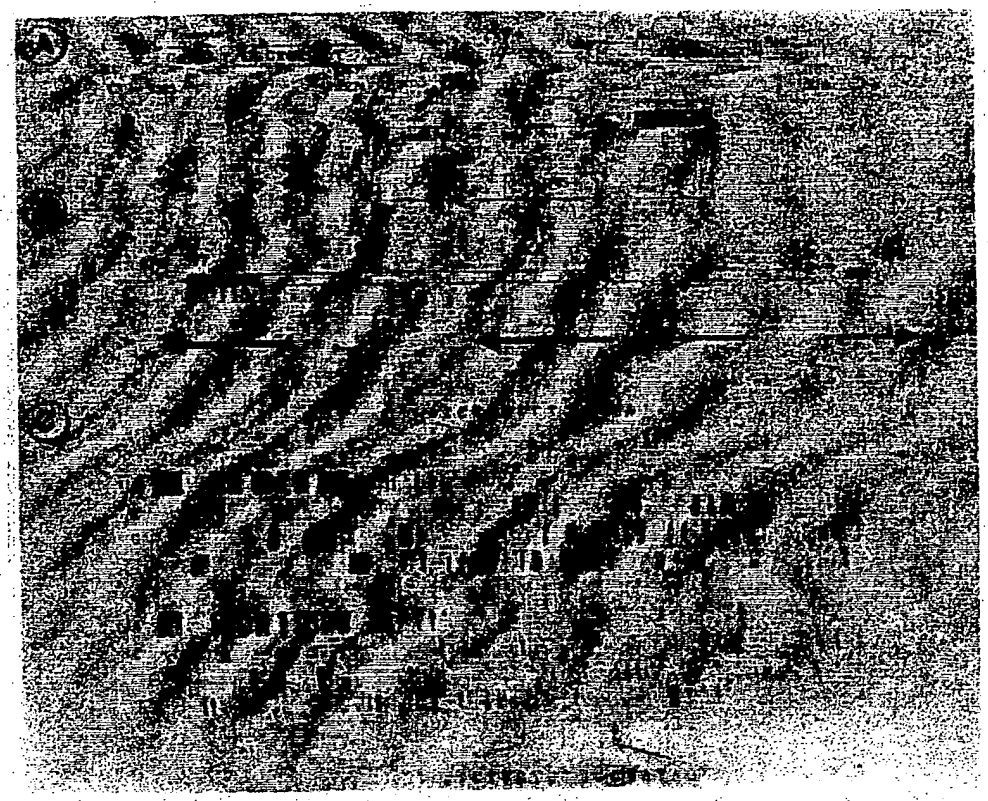


FIG. 2. Description of the SV copy-back DI H4 sequences cloned into pSP65 [pSV-DIH4A/G]. (A) Physical description of the pSP65 plasmids carrying the DI-H4 sequences. The end sequences of DI-H4, flanked by the T7 promoter and the *Bsm*I site, are boxed and written in the (−) polarity (genomic sense). T7 transcripts derived are therefore of (+) polarity. The wobble at nucleotide +1 indicated by T/C corresponds to different plasmids (pSV-DIH4/A or pSV-DIH4/G). The borders of the inverted repeat (IR) are indicated with numbers corresponding to their positions in the St genome sequence. (B) Extent of partial sequencing of the pSV-DIH4 plasmids, performed with the primers described under Materials and Methods, is indicated by bold arrows. SP6, pSP65 sequences. ST7, *Sal*I-T7 promoter. IR, inverted repeats. BB, *Bsm*I/*Bam*HI sequences. (C) Partial sequence of the template strand of pSV-DIH4/A (top) and pSV-DIH4/G (bottom) showing the +1 nucleotide of the two plasmids in the template polarity.

internal primers MVA1 and MVB1 (Table 1) flanked, but did not overlap, a unique *Xmn*I site at nt 15,641 (the 5′ end of the St genome is position 15,893). After separate RTase reactions with these primers, the resulting cDNAs were amplified with either MVA2/MVA1 and MVB2/MVB1 or MVA2/MVB1 and MVB2/MVA1 so that clones which would express both (+) and (−) RNAs from the T7 promoter could be obtained. PCR products of the expected lengths were obtained in all cases (not shown), but attempts to clone these in pSP65 as full-length constructs were unsuccessful. Examination of the rare clones obtained showed that the sequences corresponded to either the original PCR fragments or to rearranged inserts (not shown).

A slightly different approach was then used. The MV fragments were cut only with *Xmn*I, then religated, and these products were then amplified with primers T7*Sal* and *BamBsm* which correspond to the 5′ nonviral sequences of primers MVA2 and MVB2 (Table 1). As a control, the amplification of the pSV-DIH4 sequence was also attempted. Under standard conditions for Taq polymerase (see Materials and Methods), only nonspecific bands of suboptimal size were faintly visible. By using a 10% DMSO buffer (see Materials and Methods) and Taq polymerase at 16 units/100 μl, the DI H4 sequence could be amplified, but not those of MV. The AmpliTaq polymerase was then replaced by its Stoffel fragment, which, according to the manufacturer (Cetus Corp.), increases thermal stability. We hoped that higher extension temperatures would allow this polymerase to better extend through the panhandles of the template, but this was not so. Extension temperatures of 80° as opposed to 72° were similarly unsuccessful, even at very high enzyme concentration (80 units/100 μl, not shown).

A critical parameter was eventually defined which allowed successful amplification, namely, a long ramp time between the hybridization and the extension steps. Under these conditions, the extension step itself was redundant. Figure 4 shows that increasing the

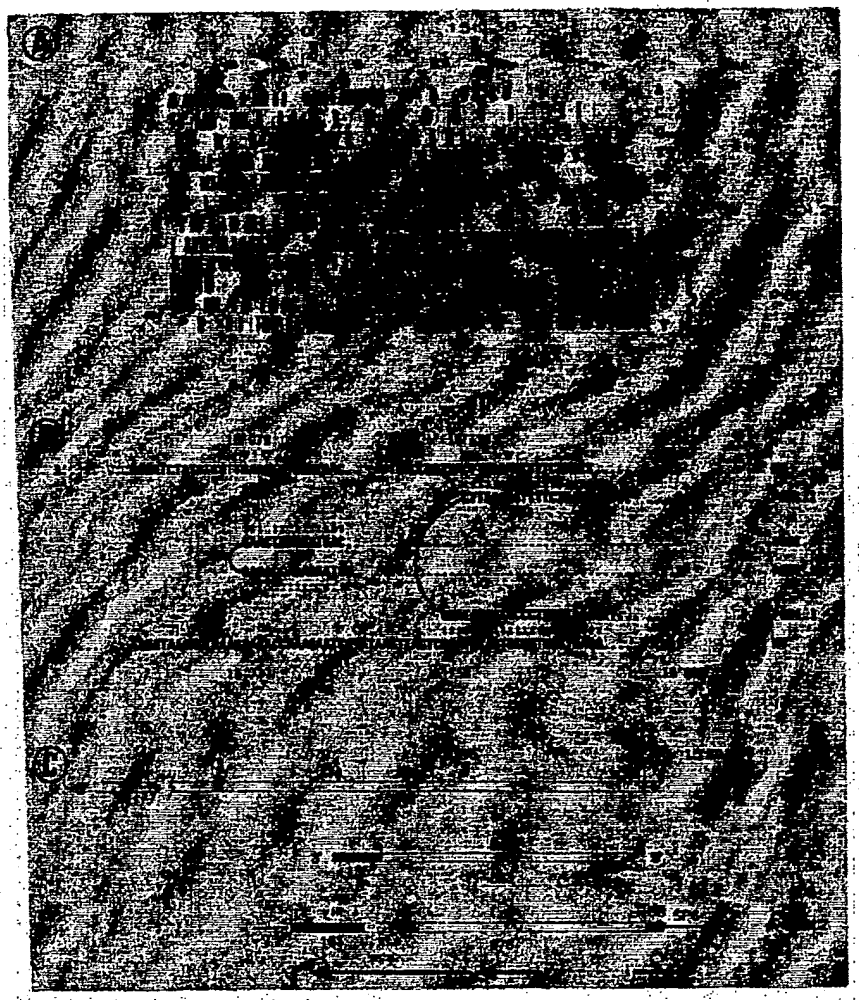


FIG. 3. Description of the different copy-back DI RNAs identified in the MV P6 DI stock. (A) PCR amplification products using a unique primer (RT, see Table 1) were obtained in identical experiments as those described in Fig. 1A using MV P6 DI RNAs. After cloning into pGem-4 plasmids and sequencing (see Materials and Methods), two different points of diverging sequences (see D in Fig. 1A) at positions 15,136 and 15,079 of the viral sequence (black arrowheads) were observed, defining two copy-back DI RNAs, P6DI.1 and P6DI.2. Open arrowheads point to nucleotide substitutions relative to the MV sequence presented at the top and obtained by sequencing plasmid pMVdelta5B (obtained from Cattaneo and Billeter, University of Zürich, see Calain and Roux, 1988). (B) Outlines of the P6DI.1 and P6DI.2 sequences in regard to the MV genomic RNAs. MV− and MV+ lines show relevant sequence stretches of MV genomic (−) and antigenomic (+) RNAs. P6DI.1 and P6DI.2 upper lines show the sequences of P6DI.1 and P6DI.2 minus strand diverging from the MV genomic sequence at the black arrowheads above MV line (positions 15,136 and 15,079 for P6DI.1 and P6DI.2, respectively). After the jump of the polymerase, presented as a curved line, the sequences of P6DI.1 and P6DI.2 (lower lines) correspond to positions 15,731 and 15,758, respectively, (black arrowheads below MV+ line) of the antigenomic MV sequence (this sequence is not presented in A). For MV−, P6DI.1, and P6DI.2, the sequences presented 3′ to 5′ are read from left to right in A. Nucleotide changes from the MV sequence are indicated by open arrowheads (see also A). (C) Schematic representation of P6DI.1 and P6DI.2 sequences. P6DI.2 is shown as present in pMV-DIP6.2(+). Bold arrows show the extent of partial sequencing performed on the full-length clones pMV-DIP6.2(+) and (−).

ramp time from 0.5 to 2.5 min resulted in a constant increase of full-length SV and MV DI DNA products. The full-length fragments, flanked by the T7 promoter and *Bsm*I sites in both orientations [designated MV-DIP6.2(+) and (−)], were then cloned into the *Sal*I/*Bam*HI sites of pSP65, and this process was now surprisingly efficient. Numerous clones were obtained ($2 \times 10^3/\mu g$ plasmid), half of them contained an insert of the correct length, and partial sequencing showed that they corresponded to the sequence shown in Fig. 3.

Expression of DI-RNAs from DNA *in vitro*

Since the cloning of copyback DI RNAs can be a problem, we examined how well the DNAs could be transcribed without the cloning step, if necessary. Us-

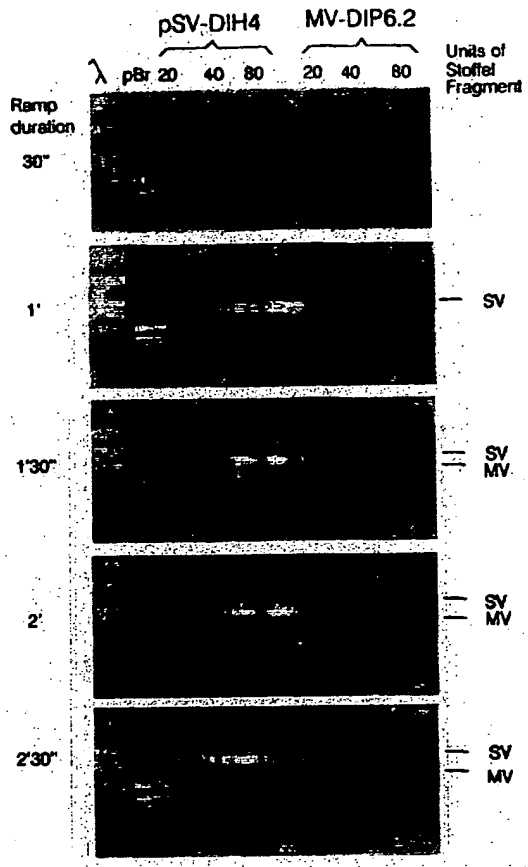


FIG. 4. PCR amplification of the complete sequence of MV P6DI.2. MVA2/A1 and MVB2/B1 products amplified in similar experiments as those described in Fig. 1B and 1C for SV were digested by *Xmn*I (see text of Results). About 20 ng of each product were mixed and ligated, and about .1 ng of the ligated product was amplified using the T7*Sal* and *BamBsm* primers (Table 1). The amplification reactions (20 μl, using the Cetus 9600 apparatus) were performed with different amounts of Stoffel fragments (20, 40, 80 units) using 30 cycles with the following parameters: a denaturation step at 96° for 10 sec, a quick cooling step to 54°, no hybridization step but a variable period of time (30 sec to 2 min and 30 sec, defined as the ramp) to reach the elongation temperature at 80° which is kept for 10 sec only. As a control the full-length sequence of SV DI-H4 was similarly amplified from equivalent amounts of the plasmid pSV-DIH4/A. The PCR products were analyzed by agarose gel electrophoresis. Lambda and pBr lanes, lambda DNA digested with *Hin*dIII/*Eco*RI and pBR322 DNA digested with *Alu*I. SV-DIH4 lanes, SV amplification products from pSV-DIH4/A. MV-P6DI.2 lanes, measles virus amplification products from ligated MVA2/A1 and MVB2/B1 products. SV and MV, respectively, SV DI-H4 and MV P6DI.2 amplified products.

ing the optimized protocol described above, measles P6DI.2 DNAs were prepared in 100-μl PCR reactions from the original ligation reactions. After polyacrylamide gel purification, the full-length products were used directly to synthesize T7 transcripts, and this was compared with those from the plasmids pMV-DIP6.2(+)/(−) and pSV-DIH4A/G. Figure 5 shows a PAGE analysis of the transcripts, and Table 2 compares the efficiencies of the various *in vitro* reactions. RNAs of the correct length were produced in all cases, and the efficiencies of transcription were not significantly different whether PCR products or plasmids were used as templates [compare in Table 2 columns MV-DIP6.2(+)/(−) with column pMV-DIP6.2(+)/(−)]. Also, pSV-DIH4/G reproducibly produced more T7 transcripts than pSV-DIH4/A, as T7 polymerase prefers to begin its chains with GTP (Yisraeli and Melton, 1989). Nevertheless, significant amounts of DI-H4 RNAs can be made which begin with ATP. The 5' ends of these latter transcripts were found to be identical to those of naturally made DI H4 RNA by primer extension analysis, and their 3' ends (after *Bsm*I linearization) were found to be predominantly U_{OH} by pCp labeling and nearest neighbor analysis (not shown).

Encapsidation and replication of SV DI-H4 RNA from DNA *in vivo*

We next examined whether the DI RNAs expressed from DNAs could be amplified by helper virus. We ex-

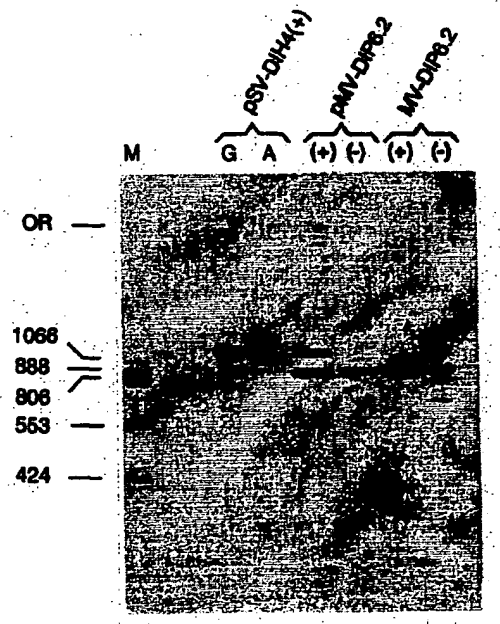


FIG. 5. PAGE analysis of the T7 transcripts synthesized from SV and MV DI plasmids or from PCR amplified DNA molecules. 2.2 μg each of pSV-DIH4A/G, 2.0 μg each of pMV-DIP6.2(+)/(−), and 0.5 μg each of the DNA representing the complete P6DI.2 sequence amplified by PCR as described in Fig. 4 [MV-DIP6.2(+)/(−)] were used to prime T7 transcription reactions in presence of [^{32}P-α]UTP. Aliquots were analyzed by electrophoresis on 4% acrylamide–urea gels. Lane M, marker lane showing T7 transcripts from the pGem4-SVM gene linearized with *Bsm*I, *Mbo*II, *Sca*I, *NCo*I, *Hae*III to give transcripts of the indicated sizes (number of nucleotides).

TABLE 2

T7 TRANSCRIPTION EFFICIENCY

	Template					
	pSV-DIH4(+)[a]		pMV-DIP6.2[b]		MV-DIP6.2[c]	
	A	G	(+)	(−)	(+)	(−)
Exp. 1	3.14[d]	14.9	ND	ND	21.9	11.5
Exp. 2	0.19	2.4	0.27	0.15	0.15	0.19

Note. ND, not done. Experiments 1 and 2 were done using T7 RNA polymerase from Biofinex (Switzerland) and from Biolabs, respectively.

[a] Transcripts synthesized from pSV-DIH4(+) plasmids starting with A or G.

[b] Transcripts of positive or negative polarity made from pMV-DIP6.2(+)/(−) plasmids.

[c] Transcripts of positive or negative polarity made from MV-DIP6.2(+)/(−) DNAs.

[d] Picomole of transcript/pmol of template. The amounts of templates were estimated by analysis of serial dilutions of the DNAs by agarose gel electrophoresis followed by ethidium bromide staining and comparison with standard amounts of DNAs (only the template portion of the plasmids were taken into account for calculation). The amounts of transcripts were estimated by the measure of [^{32}P-α]uridine incorporation.

pected that the limiting step here would be the encapsidation of the DI RNA so that it can be recognized by the viral polymerase and that this would be particularly difficult with copy-back RNAs because the long inverted repeats sequester the terminal sequences in dsRNA.

Our first attempts were to transfect *Bsm*I-linearized pSV-DIH4 into cells infected with a vaccinia virus recombinant expressing the T7 RNA polymerase and to superinfect these cells with natural St SV. The NC band region from CsCl density gradients of the resulting cell extracts was then examined by Northern blotting with a riboprobe of the same polarity [(+)] as the DI RNA made by T7 polymerase from pSV-DIH4. These attempts, however, were uniformly unsuccessful. We then replaced the natural SV superinfection with pGEM plasmids which express the NP, P/C, and L proteins via T7 polymerase, and these were cotransfected with pSV-DIH4. These pGEMs have previously been shown to provide all the functions necessary to amplify natural DI-H4 genome NCs (Curran *et al.*, 1991). This approach was successful, and one such experiment is shown in Fig. 6. A DI-H4 RNA band of (−) polarity (i.e., complementary to the T7 transcript) and expected mobility was clearly detected at 2 days postinfection and increased in intensity on Day 3. The negative control here (lane CTRL) shows that no amplification could be detected when the pGEM plasmids were omitted, and the positive control (lane DI-H4^{UV}) shows the results of omitting pSV-DIH4 and replacing it with natural but inert DI-H4 NCs (produced by UV irradiation of SV DI-H4 stock). Judging from the strength of the signal relative to the positive control, multiple rounds of replication must have occurred. Such results could be obtained with pSV-DIH4/A in four of four experiments (although generally at much lower levels than that shown in Fig. 6), and at the limit of detection in one of two experiments with pSV-DIH4/G. Moreover, visible levels of DI-H4 amplification were obtained only upon linearization of pSV-DIH4 with *Bsm*I prior to transfection.

Although the DI-H4 RNA rescued by the cloned genes was isolated by equilibrium banding on CsCl gradients (a method that allows isolation of only encapsidated viral RNAs), DI-H4 encapsidation was further investigated by measuring its sensitivity to ribonuclease, as the RNA in SV nucleocapsids is known to be resistant to RNAse digestion (Lynch and Kolakofsky, 1978). The CsCl-banded nucleocapsids were therefore treated with micrococcal nuclease before deproteinization. In conditions where deproteinized purified DI-H4 RNA was completely digested, these DI-H4 RNAs were only partially sensitive to the nuclease treatment (compare lanes 1, 2 to lanes 3, 4, Fig. 7A).

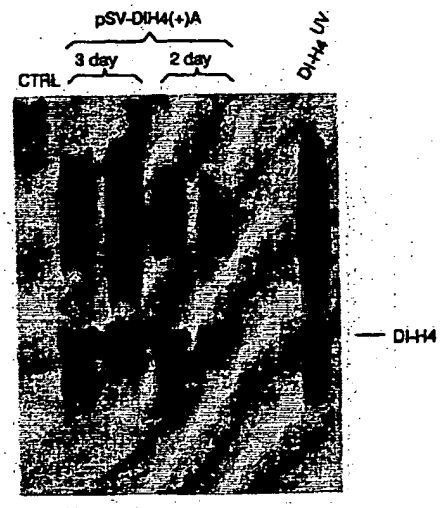


FIG. 6. Rescue of the SV DI-H4 RNA synthesized from pSV-DIH4/A by cloned L, P, and NP genes. BHK cells seeded on 9-cm petri dishes were infected with vTF7-3 at an m.o.i. of 2–3. One hour postinfection, the cells were transfected with pGem4-NP (2.5 μg), pGem4-P/C (5 μg), and pGem4-L (1.5 μg) as previously described (Curran *et al.*, 1991). At 5 hr postinfection the cells were retransfected with plasmid pSV-DIH4/A linearized with *Bsm*I (5 μg). Cytoplasmic extracts were prepared at 2 and 3 days postinfection, and isolated NC RNAs were analyzed by Northern blot using probe "5'ex" of (+) polarity (Mottet and Roux, 1990). CTRL lane represents NC RNA purified from cells transfected with only the linearized pSV-DIH4/A and harvested 3 days postinfection. As positive control, the natural DI-H4^{UV} was amplified with the cloned genes as described before (Curran *et al.*, 1991).

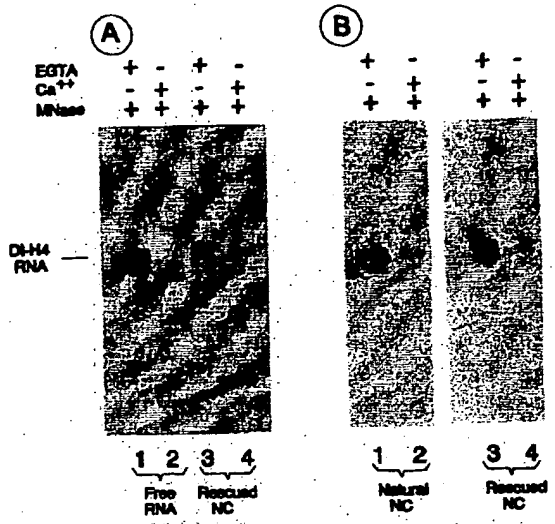


FIG. 7. Nuclease resistance of the SV DI-H4 NC RNA synthesized from pSV-DIH4/A by cloned L, P, and NP genes. (A) DI-H4 CsCl purified nucleocapsids produced by the rescue of DI-H4 RNA synthesized from pSV-DIH4/A as shown in Fig. 6 (rescued NC) were diluted sevenfold with TNE and concentrated by centrifugation (SW 55, 50 K, 1 hr, 4°) through 50% glycerol onto a cushion of 68% sucrose in D_2O. The nucleocapsids were collected at the glycerol/D_2O sucrose interface and diluted to 2 ml with TNE. Identical samples were treated with 20 μg of micrococcal nuclease (MNase) for 30 min at 37° in the presence of 20 m*M* EGTA or 2 m*M* $CaCl_2$. At the end of the incubation, 200 μl of 100 m*M* EGTA and 100 μg tRNA were added, and the samples were phenol/chloroform extracted, ethanol precipitated, and analyzed by Northern blot as in Fig. 6. In lanes 1 and 2 comparable amounts of DI-H4 nucleocapsid RNA previously deproteinized by phenol/chloroform extraction were incorporated to glycerol/D_2O interface mock solutions and treated similarly. (B) Nucleocapsids isolated from CV1 cells infected with DI-H4 (lanes 1, 2) or DI-H4 nucleocapsids rescued by the cloned genes in a separate experiment from that presented in A (lanes 3, 4) were treated exactly as in A.

This partial sensitivity, however, does not appear to be due to defective assembly of the DI RNA, as the RNA in bona fide DI-H4 nucleocapsids were also partially sensitive to micrococcal nuclease (Fig. 7B).

DISCUSSION

We at first feared that the presence of the long inverted repeats at the ends of copy-back DI genomes would make plasmids which carry these sequences unstable in *E. coli*. However, the use of the Sure strain of *E. coli* as host appears to have circumvented this problem in part. Once generated, these plasmids appear stable in this strain, and their transformation efficiency is equal to that of pBR322 (not shown). However, these constructs are only metastable, as attempts to mark them with insertions or deletions have so far been unsuccessful, due to rearrangements. The SV and MV DI genomes were cloned with slightly different approaches, for the reasons outlined above. Although we suspect that there is no general rule for dealing with the cloning of novel copy-back DI genomes, the two approaches presented here provide feasible methods for this.

The unique sequence organization of copy-back DI genomes makes it possible to specifically amplify these sequences even in the presence of a large excess of St genomes. This is done by using a pair of primers which are both of negative polarity relative to the St genome and one on either side of the crossover point used during their generation. The same method can, of course, be used to examine clinical samples as well as cell cultures suspected of containing copy-back DI genomes of otherwise unknown structure. All paramyxovirus copy-backs described to date contain various amounts of the 5′ end of the St genome plus inverted repeats of 50–200 nt. The major unknown is then the length of the 5′ end of the St genome contained in the copy-back. When examining for unknown copy-backs, one can use a series of internal A1 primers (Fig. 1A) placed at different intervals from the 5′ end of the St genome together with a single A2 terminal primer. This would help in finding longer copy-backs, and the presence or absence of PCR products when several A1 primers are used will add confidence to the result. Additional specificity can be obtained, if necessary, by using primers nested to both A1 and A2.

Of the two paramyxovirus DI genomes we cloned, only SV-DI-H4 was shown to be functional in that it could be replicated in an encapsidated form by the SV NP, P/C, and L gene products. We were only able to do this with plasmids that express these proteins and not by natural helper virus. However, others have recently expressed CAT activity from artificial DI genomes of the internal deletion type using natural helper virus superinfection (Luytjes *et al.*, 1989; Yamanaka *et al.*, 1990; Park *et al.*, 1991; Collins *et al.*, 1991; Pattnaik *et al.*, 1992). The reasons for our failure with helper virus are not yet clear and may be more apparent than real. Relative to the expression of CAT activity, Northern blotting to detect RNA directly is considerably less sensitive. Starting with natural DI NCs, we now get higher levels of DI genome amplification using cloned genes than we get with helper virus, and this enhanced ability of cloned genes to support replication may be critical when only higher levels of amplification can be scored. The addition of CAT (or anti-CAT) sequences to these constructs to provide a more sensitive marker for amplification will probably be of little value here, as these copy-back genomes are not expected to express mRNAs.

The ability to express natural DI genomes from DNA and to amplify them *in vivo* with the viral NP, P, and L proteins opens the possibility of applying reverse ge-

netics to study their replication. Moreover, as these DI genomes do not express mRNAs, genome replication per se can be examined independent of transcription. The nature and location of the *cis*-acting RNA sequences important for this process can now be explored, as can the contribution of the overall length the genome and that of the terminal repeats. For constructs which are sufficiently different in length to separate on gels, this can best be done by cotransfection, examining the ability to the two species to directly compete with each other. Moreover, the interpretation of these experiments should be more straightforward. Natural virus stocks generally represent dynamic populations of DI genomes, and it is often difficult to be sure of just how many different species are present; e.g., the MV P6 stock contains two DI genomes of ca. 1 kb rather than one, as previously noted. It may also be possible to study the budding of these genomes into virus particles upon addition of plasmids which express the remaining viral M, F, and HN genes. Such a system has been recently proven successful for VSV using natural DI genomes as well as encapsidated DI RNA transcripts originating from a cDNA clone (Pattnaik and Wertz, 1991; Pattnaik *et al.*, 1992).

ACKNOWLEDGMENTS

This work was supported by grants from the Swiss National Fund to D.K. and L.R. and by grants from the Sandoz, Ciba-Geigy and Société Académique de Genève foundations to L.R. P.C.'s salary was provided by the Shweizerische Akademie der Medizinischen Wissenschaften, the Holderbank-Stiftung, the Jangen-Pöhn-Stiftung, the Roche and the de Reuter Foundations.

REFERENCES

BARRETT, A. D. T., and DIMMOCK, N. J. (1986). *In* "Current Topics in Microbiology and Immunology" (A. Clarke, R. W. Compans, M. Cooper, H. Eisen, W. Goebel, H. Koprowski, F. Melchers, M. B. A. Oldstone, R. Rott, P. K. Vogt, H. Wagner, and I. Wilson, Eds.), 128th ed., pp. 55–84. Springer-Verlag.

CALAIN, P., and ROUX, L. (1988). Generation of measles virus defective interfering particles and their presence in a preparation of attenuated live-attenuated vaccine. *J. Virol.* 62, 2859–2866.

COLLINS, P. L., MINK, M. A., and STEC, D. S. (1991). Rescue of synthetic analogs of respiratory syncytial genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. *Proc. Natl. Acad. Sci. USA* 88, 9663–9667.

CURRAN, J., BOECK, R., and KOLAKOFSKY, D. (1991). The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. *EMBO J.* 10, 3079–3085.

ENAMI, M., KOHAMA, T., and SUGIURA, A. (1989). A measles virus subgenomic RNA: Structure and generation mechanism. *Virology* 171, 427–433.

GRUNSTEIN, M., and HOGNESS, D. S. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA* 72, 3961.

HOLLAND, J. J. (1987). "The Rhabdoviruses" (R. R. Wagner, Ed.), pp. 297–360. Plenum, New York.

HOLLAND, J. J., KENNEDY, I. T., SEMLER, B. L., JONES, C. L., ROUX, L., and GRABAU, E. A. (1980). "Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, Eds.), pp. 137–192. Plenum, New York and London.

HOLLAND, J. J., SPINDLER, K., HORODYSKI, F., GRABAU, E., NICHOL, S., and VANDEPOL, S. (1982). Rapid evolution of RNA genomes. *Science* 215, 1577–1585.

HUANG, A. S. (1987). "RNA Genetics: Variability of RNA Genomes" (E. Domingo, J. J. Holland, and P. Ahlquist, Eds.), Vol. 3, pp. 195–208. CRC Press, Boca Raton, FL.

KOGAN, S. C., DOHERTY, M., and GITSCHIER, J. (1987). An improved method for prenatal diagnosis of genetics diseases by analysis of amplified DNA sequences. *N. Engl. J. Med.* 317, 985–990.

KOLAKOFSKY, D. (1976). Isolation and characterization of Sendai virus DI-RNAs. *Cell* 8, 547–555.

LEPPERT, M., KORT, L., and KOLAKOFSKY, D. (1977). Further characterization of Sendai virus DI-RNAs: A model for their generation. *Cell* 12, 539–552.

LUYTJES, W., KRYSTAL, M., ENAMI, M., PARVIN, J. D., and PALESE, P. (1989). Amplification, expression, and packaging of a foreign gene by influenza virus. *Cell* 59, 1107–1113.

LYNCH, S., and KOLAKOFSKY, D. (1978). The ends of the RNA within Sendai virus defective interfering nucleocapsids are not free. *J. Virol.* 28, 584–589.

MOTTET, G., and ROUX, L. (1990). Budding efficiency of Sendai virus nucleocapsids: Influence of size and ends of the RNA. *Virus Res.* 14, 175–188.

PARK, K. H., HUANG, T., CORREIA, F. F., and KRYSTAL, M. (1991). Rescue of a foreign gene by Sendai virus. *Proc. Natl. Acad. Sci. USA* 88, 5537–5541.

PATTNAIK, A. K., and WERTZ, G. W. (1991). Cells that express all five proteins of vesicular stomatitis virus from cloned cDNAs support replication, assembly, and budding of defective interfering particles. *Proc. Natl. Acad. Sci. USA* 88, 1379–1383.

PATTNAIK, A. K., BALL, L. A., LEGRONE, A. W., and WERTZ, G. W. (1992). Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* 69, 1011–1020.

PERRAULT, J. (1981). Origin and replication of defective interfering particles. *Curr. Top. Microbiol. Immunol.* 93, 151–207.

RE, G. G. (1991). "The Paramyxoviruses" (D. W. Kingsbury, Ed.), pp. 275–298. Plenum, New York and London.

ROUX, L., SIMON, A. E., and HOLLAND, J. J. (1991). "Advances in Virus Research." Vol. 40, pp. 181–211. Academic Press, San Diego.

SCHLESINGER, S. (1987). "RNA Genetics: Retroviruses, Viroids, and RNA Recombination" (E. Domingo, J. J. Holland, and P. Ahlquist, Eds.), pp. 167–185. CRC Press, Boca Raton, FL.

YAMANAKA, K., ISHIHAMA, A., and NAGATA, K. (1990). Reconstitution of influenza virus RNA–nucleoprotein complexes structurally resembling native viral ribonucleoprotein cores. *J. Biol. Chem.* 265, 1151–1155.

YISRAELI, J. K., and MELTON, D. A. (1989). Synthesis of long, capped transcripts *in vitro* by SP6 and T7 RNA polymerases. *Methods Enzymol.* 180, 42–50.

Volume 14 Number 4 1986 Nucleic Acids Research

Determination of the complete nucleotide sequence of the Sendai virus genome RNA and the predicted amino acid sequences of the F, HN and L proteins

Tatsuo Shioda, Kentaro Iwasaki[1] and Hiroshi Shibuta*

Department of Viral Infection, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108 and [1]Department of Physiological Chemistry, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan

Received 14 January 1986; Accepted 24 January 1986

ABSTRACT

We previously determined the 3' proximal 5,824 nucleotides of the Sendai virus genome RNA (Nucleic Acids Res. 11, 7317-7330, 1983; Nucleic Acids Res. 12, 7965-7973, 1984), and present here the sequence of the remaining 5' proximal 9,559 nucleotides. Thus, this is the first paramyxovirus to have its genome organization elucidated. The set of complementary DNA clones used was prepared by the method of Okayama and Berg from polyadenylylated viral genome RNA. We sequenced the region containing the 5' proximal half of the F gene, and the subsequent HN and L genes, and predicted the complete amino acid sequence of the products of these genes. Sequence analyses confirmed that all the genes are flanked by consensus sequences and suggest that the viral mRNAs are capable of forming stem-and-loop structures. Comparison of the F and HN glycoproteins of Sendai virus with those of simian virus 5 strongly suggests that the cysteine residues are highly important for maintenance of the molecular structures of these glycoproteins.

INTRODUCTION

Recently, the molecular cloning of complementary DNA (cDNA) to RNA has greatly contributed to the elucidation of the gene and genome structures of several RNA viruses. Analyses of the viral gene and genome structures provide essential information for the solving of several important problems such as the mechanisms of viral replication and transcription, the nature of each viral protein, biological events caused by viruses and genetical relationships among viruses.

Of paramyxoviruses, Sendai virus has been most extensively studied as a prototype, its genome being a single continuous RNA of about 15 kilobases long with negative polarity. Sendai virus contains six structural proteins, i.e. large (L), RNA polymerase (P), nucleocapsid (NP), hemagglutinin-neuraminidase (HN), fusion (F) and membrane (M) proteins. In addition, at least one non-structural viral protein designated as C (1) and a small transcript called leader RNA (2) have been identified in infected cells. In previous communications (3,4), we reported the sequence of the 5,824

nucleotides from the 3' end of the Sendai virus genome RNA and determined the complete primary structures of the first three genes and a part of the fourth gene. Our results as well as data presented by others established that the gene order is 3'-leader-NP-P+C-M-F- (3,4,5,6,7,8,9), and the subsequent gene order has been proposed to be -HN-L-5' (10,11). In order to fully elucidate the genome structure of Sendai virus, we continued to construct cDNA clones toward the 5' end, and present here the full sequence of the remaining region, that is, the 5' proximal 9,559 nucleotides, which contains the 5' proximal half of the F gene and the subsequent HN and L genes. Thus, we have determined the complete primary structure of the genome of Sendai virus strain Z, and have predicted the amino acid sequence of each gene product. We also compare the predicted structures of the F and HN glycoproteins of Sendai virus with those reported for another paramyxovirus, simian virus 5 (SV5) (12,13).

MATERIALS AND METHODS

Preparation of viral RNA

Sendai virus strain Z was used. Viral 50S genome RNA was prepared from virions purified from infectious allantoic fluids of chicken eggs as described previously (3). Sendai virus mRNAs were prepared in a similar way to as described previously (3) using BHK-21 cells as the host cells, and RNA was extracted by the guanidium thiocyanate method (14). The mRNAs were selected from crude RNA by oligo(dT) cellulose column chromatography.

Synthesis and cloning of the complementary DNA (cDNA)

Sendai virus genome RNA was polyadenylylated according to Inokuchi et al. (15) except that the amount of ATP:RNA adenyltransferase (poly A polymerase) was increased to 20-fold. Synthesis and molecular cloning of cDNA from the polyadenylylated genome RNA were performed according to Okayama and Berg using a pSV7186-derived vector-primer (16). E. coli K12 strain HB101 was transformed with the resulting recombinant plasmids by the standard method (17).

Sequence determination of cDNA

CDNAs were cleaved into fragments with appropriate restriction endonucleases, and after subcloning of the fragments into M13 phage (18), their nucleotide sequences were determined by the method of Sanger et al.(19).

Colony hybridization

Bacterial colonies grown on nitrocellulose filters were lysed and fixed

according to Grunstein and Hogness (20). The nitrocellulose filters were used for hybridization (21) with the viral 50S RNA probe labeled with [γ-^{32}P] ATP (22) or the cDNA probe labeled with [α-^{32}P] dGTP (23).

Northern blot hybridization

Viral mRNA resolved in an agarose gel was transferred to a nitrocellulose filter as described previously (3). Hybridization of this filter with ^{32}P-labeled cDNA was performed as described above.

Enzymes and other materials

ATP:RNA adenyltransferase, E. coli DNA ligase, terminal deoxynucleotidyl transferase and ribonuclease H were purchased from P-L Biochemicals, Milwaukee, U.S.A.; avian myeloblastosis virus reverse transcriptase from Seikagaku Kogyo, Tokyo, Japan; M13 cloning and sequencing kits, nick-translation kits and all the radioactive compounds from Amersham Incorporation plc, Amersham, England; HAWP nitrocellulose membrane filters from Millipore, Bedford, U.S.A.; and bovine alkaline phosphatase, T4 polynucleotide kinase, E. coli DNA polymerase I, the Klenow fragment of DNA polymerase I, T4 DNA ligase and all the restriction endonucleases from Takara Shuzo, Kyoto, Japan.

RESULTS

Cloning and sequence determination of cDNA

We previously described the molecular cloning and sequencing of the Sendai virus genome cDNAs which cover the 3' proximal 5,824 nucleotides (3,4). In order to obtain cDNA clones which represent the remaining genome region, we tried to clone cDNAs which were prepared by reverse-transcription of partially digested and then in vitro polyadenylylated viral genome RNA. For this, 6 micrograms of Sendai virus genome RNA was incubated with 4 units of poly A polymerase, which is about 20-fold the amount used for the standard polyadenylation reaction. After the incubation, the 50S genome RNA was found to have been digested to an average size of about 28S (data not shown), which was most likely caused by the ribonuclease activity contaminating the poly A polymerase preparation. Subsequent synthesis and molecular cloning of cDNAs from the polyadenylylated RNA were performed according to Okayama and Berg (16).

Transformation of E. coli HB101 with the recombinant plasmids yielded about 500 ampicillin-resistant colonies, from which 41 were selected on the basis of the intense hybridization signals with the Sendai virus genome RNA probe. Out of these clones, 27, which did not hybridize with probes of cDNA

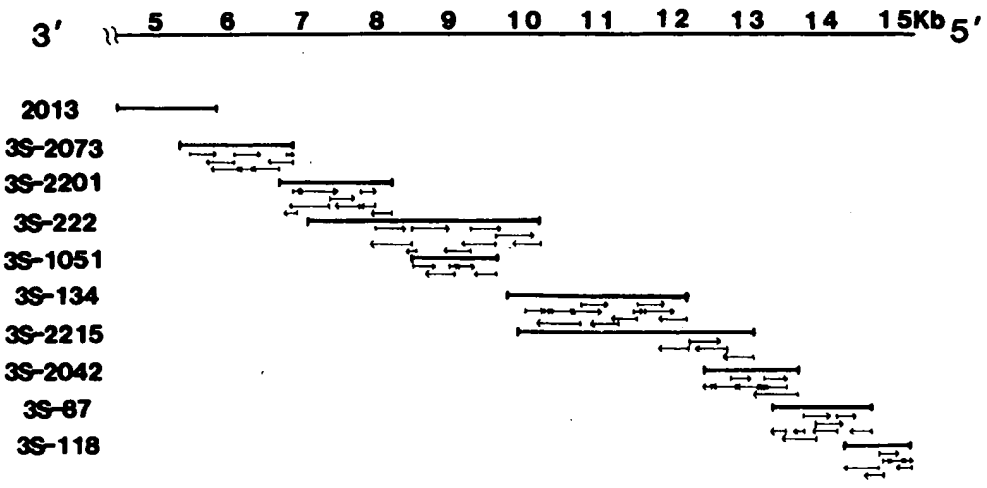


Fig. 1. Locations of previously obtained cDNA clone 2013 and newly prepared cDNA clones 3S-2073, 3S-2201, 3S-222, 3S-134, 3S-1051, 3S-2215, 3S-2042, 3S-87 and 3S-118 in the genome RNA and the sequencing strategy. Arrows indicate the cDNA fragments sequenced and the direction of sequencing.

fragments corresponding to the NP, P+C or M genes (3,4), were selected by the colony hybridization technique. The mutual relationships among the 27 clones as well as their relationships to the previously reported clones were determined by colony hybridization tests using appropriate cDNA probes in combination with restriction map analyses, and it was found that these clones could be lined without any gap in order from the 3' proximity to the 5' proximity. We selected 9 clones for sequencing. The sequential relationships between these 9 clones and to the previously reported clone 2013 together with the sequencing strategy are shown in Fig. 1. Clone 3S-2073 showed an overlap of about 400 nucleotides with clone 2013, which had been shown to cover the 5' proximal 331 nucleotides of the M gene and the 3' proximal 1,013 nucleotides of the F gene (4). Clone 3S-118, which was identified as the most 5' proximal clone, had an identical sequence with the reported 5' terminal 70 nucleotides of the Sendai virus genome RNA (24) except for 3 substitutions, which was followed by 6 guanine residues and a PstI site derived from the linker DNA fragment. This indicates that 3S-118 included the very 5' end portion of the genome, and that we had obtained a set of cDNA clones that covered the entire genome RNA. By analyzing these 9 clones, we determined the sequence of the 9,559 nucleotides of the Sendai virus genome, which followed the 3' proximal 5,824 nucleotides that we had previously determined (3,4). Thus, we concluded that the total length of

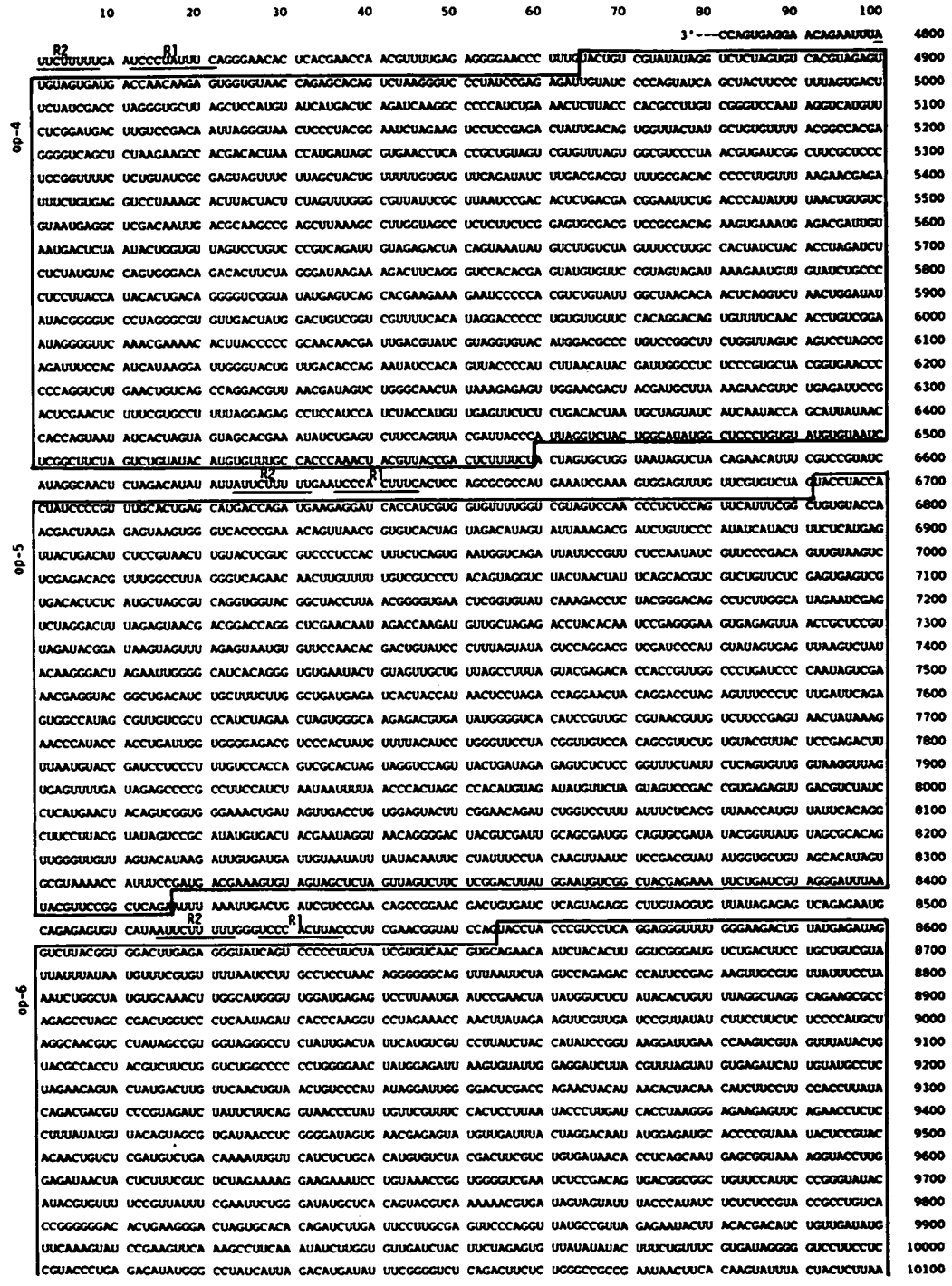
the Sendai virus genome RNA is 15,383 nucleotides, which is in good agreement with the previous value estimated from the sedimentation coefficient obtained with a sucrose density gradient (25,26). The nucleotide sequence from position 4,781, the 5' end portion of the M gene, to 15,383, the 5' end of the genome RNA, is shown in Fig. 2.

Analysis of the nucleotide sequence

Distribution of the translation termination codons in the plus strand (complementary strand to the genome) of the above region is shown in Fig.3, indicating the presence of three large open reading frames. Open reading frame op-4, which starts from nucleotide position 4,865 and encodes the F protein as reported previously (4), was found to terminate at nucleotide position 6,559, encoding a polypeptide of 565 amino acids with a molecular weight of 61,495. Downstream of op-4, two open reading frames, designated as op-5 (from 6,692 to 8,416) and op-6 (from 8,555 to 15,238), were observed. Op-5 corresponds to 575 amino acids, the calculated molecular weight of which is 63,408, and op-6 encodes a giant polypeptide of 2,228 amino acids with a calculated molecular weight of 252,864.

Previous studies (3,4,5,6,7,8) showed that the Sendai virus NP, P+C and M genes are flanked by consensus sequence R1 (UCCCACUUUC or UCCCAGUUUC) at the 3' end and by R2 (AUUCUUUUU) at the 5' end, and that the junctiuon between these genes is composed of R2-GAA-R1. This structure was also found for op-4, op-5 and op-6, i.e. they are preceded by UCCCUAUUUC, UCCCACUUUC and UCCCACUUAC, respectively, which were assigned for R1, and followed by AUUCUUUUU (R2), and the junction between R1 and R2 was GAA or GGG. Thus, we assigned the sequences from R1 starting at position 4,812 to R2 ending at 6,632, from 6,636 to 8,523 and from 8,527 to 15,326 as the fourth, fifth and sixth genes, respectively, and GAA as the intergenic sequence with one exception between the fifth and sixth genes where it was -GGG-. It is of interest to note that the sequence, -GAA-, was again found after R2 of the last gene.

Since we have already assigned the first to fourth genes for NP, P+C, M and F, respectively, the fifth and sixth genes correspond to the HN and L genes. The coding capacity of op-5, 63,408, is very close to the reported value for the molecular weight of the unglycosylated form of the HN protein of 63K (27,28), while that of op-6, 252,864, is very similar to the estimated molecular weight of the L protein of about 200K (29). In order to confirm that op-6 really produces a large transcript, northern blot hybridization was performed between mRNAs from infected cells and a probe,


10
20
30
40
50
60
70
80
90
100
3'---CCAGUGAGGA ACAGAAUUUA 4800
R2
R1
UUCUUUUUGA AUCCCUAUUU CAGGGAACAC UCACGAACCA ACGUUUUGAG AGGGGAACCC UUUGUACUGU CGUAUAUAGG UCUCUAGUGU CACGUAGAGU 4900
UGUAGUGAUG ACCAACAAGA GUGGUGUAAC CAGAGCACAG UCUAAGGGUC CCUAUCCGAG AGAUUGUAUC CCCAGUAUCA GCUACUUCCC UUUAGUGACU 5000
UCUAUCGACC UAGGGUGCUU AGCUCCAUGU AUCAUGACUC AGAUCAAGGC CCCCAUCUGA AACUCUUACC CACGCCUUGU CGGGUCCAAU AGGUCAUGUU 5100
CUCGGAUGAC UUGUCCGACA AUUAGGGUAA CUCCCUACGG AAUCUAGAAG UCCUCCGAGA CUAUUGACAG UGGUUACUAU GCUGUGUUUU ACGGCCACGA 5200
GGGGUCAGCU CUAAGAAGCC ACGACACUAA CCAUGAUAGC GUGAACCUCA CCGCUGUAGU CGUGUUUAGU GGCGUCCCUA ACGUGAUCGG CUUCGCUCCC 5300
UCCGGUUUUC UCUGUAUCGC GAGUAGUUUC UUAGCUACUG UUUUUGUGUG UUCAGAUAUC UUGACGACGU UUUGCGACAC CCCCUUGUUU AAGAACGAGA 5400
UUUCUGUGAG GUCCUAAAGC ACUUACUACU CUAGUUUGGG CGUUAUUCGC UUAAUCCGAC ACUCUGACGA CGGAAUUCUG ACCCAUAUUU UAACUGUGUC 5500
GUAAUGAGGC UCGACAAUUG ACGCAAGCCG AGCUUAAAGC CUUGGUAGCC UCUCUUCUCG GAGUGCGACG UCCGCGACAG AAGUGAAAUG AGACGAUUGU 5600
AAUGACUCUA AUACUGGUGU UAGUCCUGUC CCGUCAGAUU GUAGAGACUA CAGUAAAUAU GUCUUGUCUA GUUUCCUUGC CACUAUCUAC ACCUAGAUCU 5700
CUCUAUGUAC CAGUGGGACA GACACUUCUA GGGAUAAGAA AGACUUCAGG GUCCACACGA GUAUGUGUUC CGUAGUAGAU AAAGAAUGUU GUAUCUGCCC 5800
CUCCUUACCA UACACUGACA GGGGUCGGUA UAUGAGUCAG CACGAAGAAA GAAUCCCCCA CGUCUGUAUU GGCUAACACA ACUCAGGUCU AACUGGAUAU 5900
AUACGGGGUC CCUAGGGCGU GUUGACUAUG GACUGUCGGU CGUUUUCACA UAGGACCCCC UGUGUUGUUC CACAGGACAG UGUUUUCAAC ACCUGUCGGA 6000
AUAGGGGUUC AAACGAAAAC ACUUACCCCC GCAACAACGA UUGACGUAUC GUAGGUGUAC AUGGACGCCC UGUCCGGCUU CUGGUUAGUC AGUCCUAGCG 6100
AGAUUUCCAC AUCAUAAGGA UUGGGUACUG UUGACACCAG AAUAUCCACA GUUACCCCAU CUUAACAUAC GAUUGGCCUC UCCCGUGCUA CGGUGAACCC 6200
CCCAGGUCUU GAACUGUCAG CCAGGACGUU AACGAUAGUC UGGGCAACUA UAAAGAGAGU UGGAACGACU ACGAUGCUUA AAGAACGUUC UGAGAUUCCG 6300
ACUCGAACUC UUUCGUGCCU UUUAGGAGAG CCUCCAUCCA UCUACCAUGU UGAGUUCUCU CUGACACUAA UGCUAGUAUC AUCAAUACCA GCAUUAUAAC 6400
CACCAGUAAU AUCACUAGUA GUAGCACGAA AUAUCUGAGU CUUCCAGUUA CGAUUACCCA UUAGGUCUAC UGGCAUAUGG CUCCCUGUGU AUGUGUAAUC 6500
UCGGCUUCUA GUCUGUAUAC AUGUGUUUGC CACCCAAACU ACGUUACCGA CUCUUUUCUA CUAGUGCUGG UAAUAGUCUA CAGAACAUUU CGUCCGUAUC 6600
R2
R1
AUAGGCAACU CUAGACAUAU AUUAUUCUUU UUGAAUCCCA CUUUCACUCC AGCGCGCCAU GAAAUCGAAA GUGGAGUUUG UUCGUGUCUA CUACCUACCA 6700
CUAUCCCCGU UUGCACUGAG CAUGACCAGA UGAAGAGGAU CACCAUCGUG GUGUUUUGGU CGUAGUCCAA CCCUCUCCAG UUCAUUUCGC CUGUGUACCA 6800
ACGACUAAGA GAGUAAGUGG GUCACCCGAA ACAGUUAACG GUGUCACUAG UAGACAUAGU AUUAAAGACG AUCUGUUCCC AUAUCAUACU UUCUCAUGAG 6900
UUACUGACAU CUCCGUAACU UGUACUCGUC GUCCCUCCAC UUUCUCAGUG AAUGGUCAGA UUAUUCCGUU CUCCAAUAUC GUUCCCGACA GUUGUAAGUC 7000
UCGAGACACG UUUGGCCUUA GGGUCAGAAC AACUUGUUUU UGUCGUCCCU ACAGUAGGUC UACUAACUAU UCAGCACGUC GUCUGUUCUC GAGUGAGUCG 7100
UGACACUCUC AUGCUAGCGU CAGGUGGUAC GGCUACCUUA ACGGGGUGAA CUCGGUGUAU CAAAGACCUC UACGGGACAG CCUCUUGGCA UAGAAUCGAG 7200
UCUAGGACUU UAGAGUAACG ACGGACCAGG CUCGAACAAU AGACCAAGAU GUUGCUAGAG ACCUACACAA UCCGAGGGAA GUGAGAGUUA ACCGCUCCGU 7300
UAGAUACGGA UAAGUAGUUU AGAGUAAUGU GUUCCAACAC GACUGUAUCC CUUUAGUAUA GUCCAGGACG UCGAUCCCAU GUAUAGUGAG UUAAGUCUAU 7400
ACAAGGGACU AGAAUUGGGG CAUCACAGGG UGUGAAUACU GUAGUUGCUG UUAGCCUUUA GUACGAGACA CCACCGUUGG CCCUGAUCCC CAAUAGUCGA 7500
AACGAGGUAC GGCUGACAUC UGCUUUCUUG GCUGAUGAGA UCACUACCAU AACUCCUAGA CCAGGAACUA CAGGACCUAG AGUUUCCCUC UUGAUUCAGA 7600
GUGGCCAUAG CGUUGUCGCU CCAUCUAGAA CUAGUGGGCA AGAGACGUGA UAUGGGUCA CAUCCGUUGC CGUAACGUUG UCUUCCGAGU AACUAUAAAG 7700
AACCCAUACC ACCUGAUUGG UGGGGAGACG UCCCACUAUG UUUUACAUCC UGGGUUCCUA CGGUUGUCCA CAGCGUUCUG UGUACGUUAC UCCGAGACUU 7800
UUAAUGUACC GAUCCUCCCU UUGUCCACCA GUCGCACUAG UAGGUCCAGU UACUGAUAGA GAGUCUCUCC GGUUUCUAUU CUCAGUGUUG GUAAGGUUAG 7900
UCAGUUUUGA UAGAGCCCCG CCUUCCAUCU AAUAAUUUUA ACCCACUAGC CCACAUGUAG AUAUGUUCUA GUAGUCCGAC CGUGAGAGUU GACGUCUAUC 8000
CUCAUGAACU ACAGUCGGUG GGAAACUGAU AGUUGACCUG UGGAGUACUU CGGAACAGAU CUGGUCCUUU AUUUCUCACG UUAACCAUGU UAUUCACAGG 8100
CUUCCUUACG UAUAGUCCGC AUAUGUGACU ACGAAUAGGU AACAGGGGAC UACGUCGAUU GCAGCGAUGG CAGUGCGAUA UACGGUUAUG UAGCGCACAG 8200
UUGGGUUGUU AGUACAUAAG AUUGUGAUGA UUGUAAUAUU UAUACAAUUC CUAUUUCCUA CAAGUUAAUC UCCGACGUAU AUGGUGCUGU AGCACAUAGU 8300
GCGUAAAACC AUUUCCGAUG ACGAAAGUGU AGUAGCUCUA GUUAGUCUUC UCGGACUUAU GGAAUGUCGG CUACGAGAAA UUCUGAUCGU AGGGAUUUAA 8400
UACGUUCCGG CUCAGAUUUU AAAUUGACUG AUCGUCCGAA CAGCCGGAAC GACUGUGAUC UCAGUAGAGG CUUGUAGGUG UUAUAGAGAG UCAGAGAAUG 8500
R2
R1
CAGAGAGUGU CAUAAUUCUU UUUGGGUCCC ACUUACCCUU CGAACGGUAU CCAGUACCUA CCCGUCCUCA GGAGGGUUUU GGGAAGACUG UAUGAGAUAG 8600
GUCUUACGGU GGACUUGAGA GGGUAUCAGU CCCCCUUCUA UCGUGUCAAC GUGCAGAACA AUCUACACUU GGUCGGGAUG UCUGACUUCC UGCUGUCGUA 8700
UUAUUUAUAA UGUUUCGUGU UUUAAUCCUU GCCUCCUAAC AGGGGGGCAG UUUAAUUCUA GUCCAGAGAC CCAUUCCGAG AAGUUGCGUG UUAUUUCCUA 8800
AAUCUGGCUA UGUGCAAACU UGGCAUGGGU UGGAUGAGAG UCCUUAAUGA AUCCGAACUA UAUGGUCUCU AUACACUGUU UUAGGCUAGG CAGAAGCGCC 8900
AGAGCCUAGC CGACUGGUCC CUCAAUAGAU CACCCAAGGU CCUAGAAACC AACUUAUAGA AGUUCGUUGA UCCGUUAUAU CUUCCUUCUC UCCCCAUGCU 9000
AGGCAACGUC CUAUAGCCGU GGUAGGGCCU CUAUUGACUA UUCAUGUCGU CCUUAUCUAC CAUAUCCGGU AAGGAUUGAA CCAAGUCGUA GUUUAUACUG 9100
UACGCCACCU ACGUCUUCUG GUCUGGCCCC CCUGGGGAAC UAUGGAGAUU AAGUGUAUUG GAGGAUCUUA CGUUUAGUAU GUGAGAUCAU UGUAUGCCUC 9200
UAGAACAGUA CUAUGACUUG UUCAACUGUA ACUGUCCCAU AUAGGAUUGG GGACUCGACC AGAACUACAU AACACUACAA CAUCUUCCUU CCACCUUAUA 9300
CAGACGACGU CCCGUAGAUC UAUUCUUCAG GUAACCCUAU UGUUCGUUUC CACUCCUUAA UACCCUUGAU CACCUAAGGG AGAAGAGUUC AGAACCUCUC 9400
CUUUAUAUGU UACAGUAGCG UGAUAACCUC GGGGAUAGUG AACGAGAGUA UGUUGAUUUA CUAGGACAAU AUGGAGAUGC ACCCCGUAAA UACUCCGUAC 9500
ACAACUGUCU CGAUGUCUGA CAAAAUUGUU CAUCUCUGCA CAUGUGUCUA CGACUUCGUC UGUGAUAACA CCUCAGCAAU GAGCGGUAAA AGGUACCUUG 9600
GAGAUAACUA CUCUUUCGUC UCUAGAAAAG GAAGAAAUCC UGUAAACCGG UGGGGUCGAA UCUCCGACAG UGACGGCGGC UGUUCCAUUC CCGGGUAUAC 9700
AUACGUGUUU UCCGUUAUUU CGAAUUCUGG GAUAUGCUCA CAGUACGUCA AAAAACGUGA UAGUAGUAUU UACCCAUAUC UCUCUCCGUA CCGCCUGUCA 9800
CCGGGGGGAC ACUGAAGGGA CUAGUGCACA CAGAUCUUGA UUCCUUGCGA GUUCCCAGGU UAUGCCGUUA GAGAAUACUU ACACGACAUC UGUUGAUAUG 9900
UUCAAAGUAU CCGAAGUUCA AAGCCUUCAA AUAUCUUGGU GUUGAUCUAC UUCUAGAGUG UUAUAUAUAC UUUCUGUUUC GUGAUAGGGG GUCCUUCCUC 10000
CGUACCCUGA GACAUAUGGG CCUAUCAUUA GACAUGAUAU UUCGGGGUCU CAGACUUCUC UGGGCCGCCG AAUAACUUCA CAAGUAUUUA CUACUCUUAA 10100
op-4
op-5
op-6

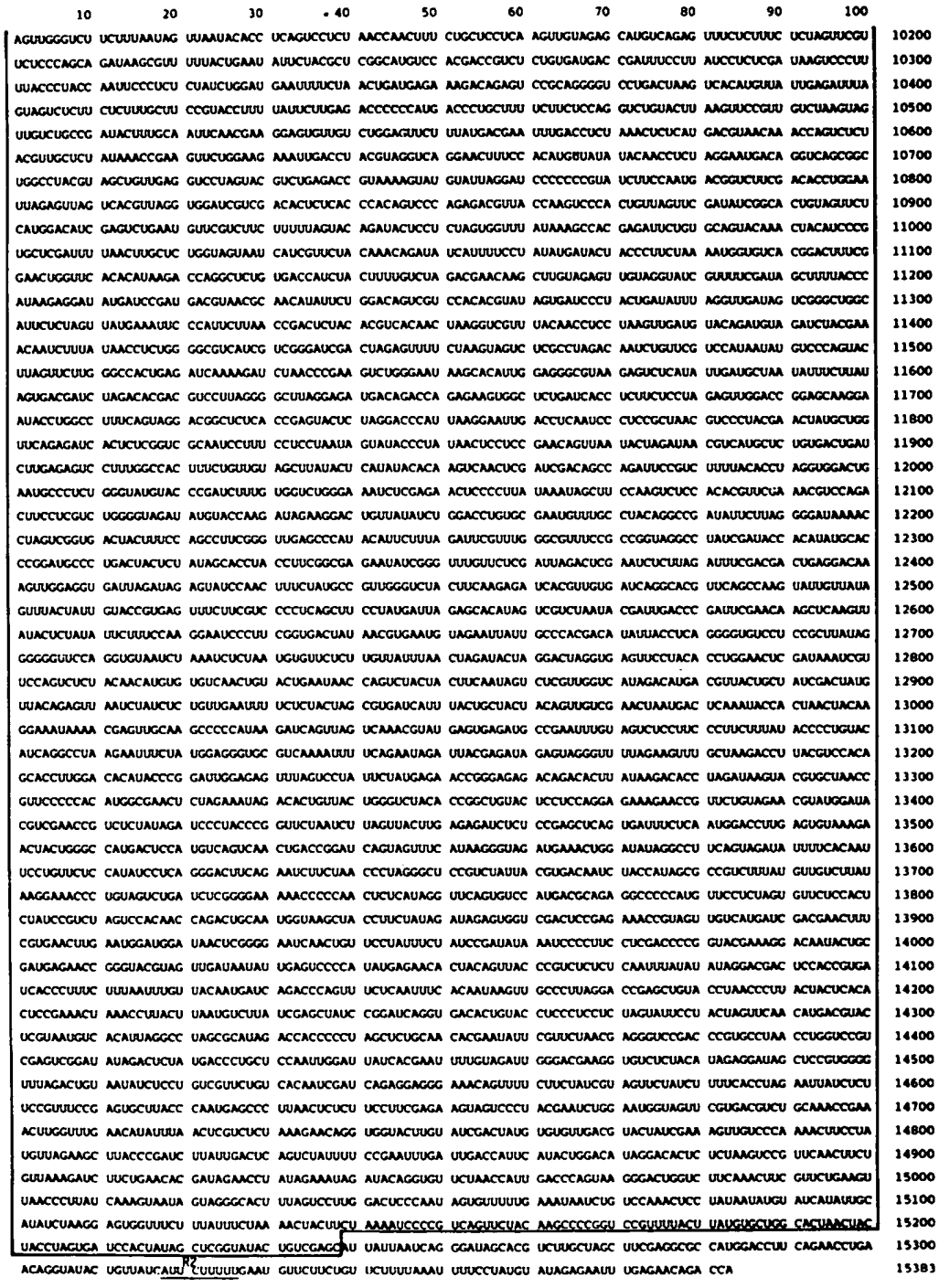


Fig. 2. The RNA sequence of the Sendai virus genome (Z strain) from nucleotide position 4,781 to 15,383, the 5' end of the genome. R1 and R2 are the repeating consensus sequences. Op-4, op-5 and op-6 denote the large open reading frames.

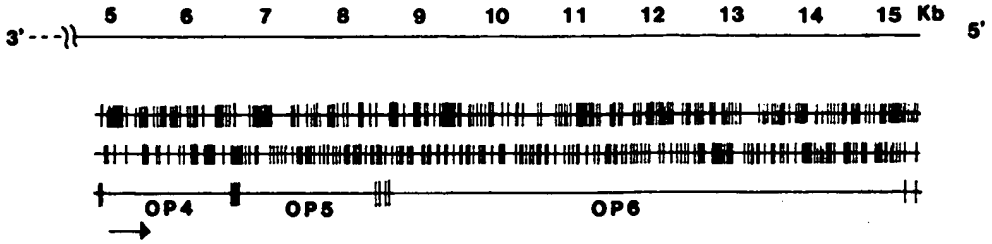


Fig. 3. Distribution of translation termination codons (vertical bars) in the plus strand RNA of the genome region presented in Fig. 2. An arrow indicates the direction of translation.

namely a cDNA fragment correponding to the 3' end portion of op-6 (from 8,540 to 9,719), which was prepared from clone 3S-222 by digestion with HindIII, followed by labeling in vitro by nick-translation. This probe hybridized with the largest virus specific poly(A) RNA as shown in Fig. 4. From these observations, we concluded that op-5 and op-6 encode the HN and L proteins, respectively.

No open reading frame was detected within the sequence of the 5' terminal 54 nucleotides following -GAA after the last gene, and the 3' half of this sequence was found to be U-rich and the 5' half to be A-rich. Thus, this region may be considered as the 5' leader region as reported for vesicular stomatitis virus (VSV). It is interesting to note that the most 5' terminal 12 nucleotides of this region are complementary (including one wobbling base pair) to the most 3' terminal 12 nucleotides of the genome,

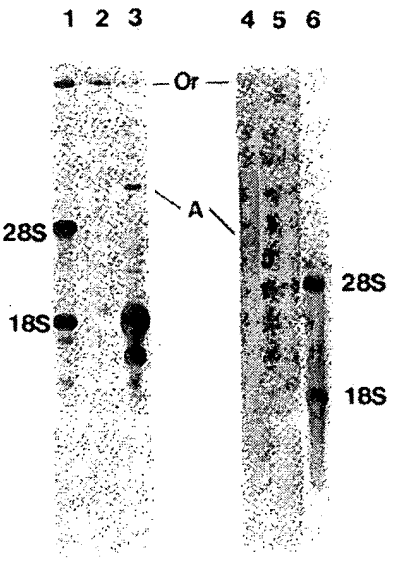


Fig.4. Left: Agarose gel electrophoresis of ^{32}P-labeled mRNAs from Sendai virus infected BHK-21 cells (lane 3) and those from uninfected cells (lane 2). Right: Hybridization of ^{32}P-labeled cDNA corresponding to the 3' end portion of OP-6 (nucleotide position 8,540 to 9,721) with mRNAs from infected cells (lane 4) and uninfected cells (lane 5). ^{32}P-labeled ribosomal RNA from uninfected cells served as size markers (lanes 1 and 6). "A" indicates the largest mRNA. "Or" means the origin.

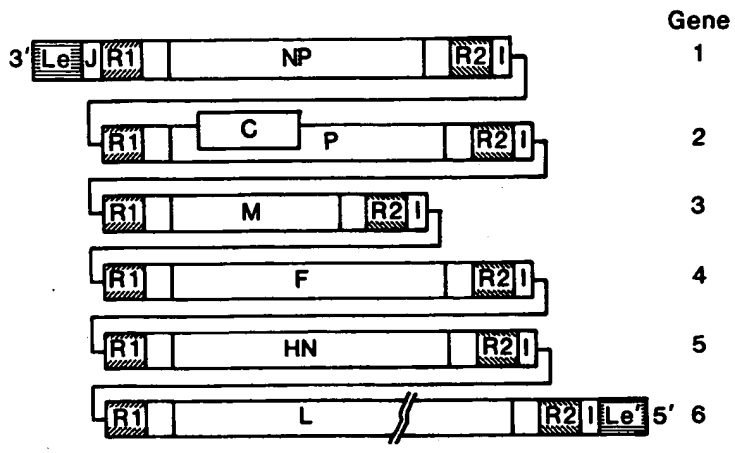


Fig. 5. A schematic illustration of the entire Sendai virus genome RNA. Le and Le' indicate the 3' leader region and the putative 5' leader region, respectively. I is the sequence, GAA or GGG, and J the sequence, AAAA.

which is in good agreement with a previous observation by Re et al.(24). This observation suggests the possibility that both ends of the genome RNA construct a stable secondary structure, giving the genome RNA a panhandle structure.

Combining the present results with the previous ones (3,4), we present

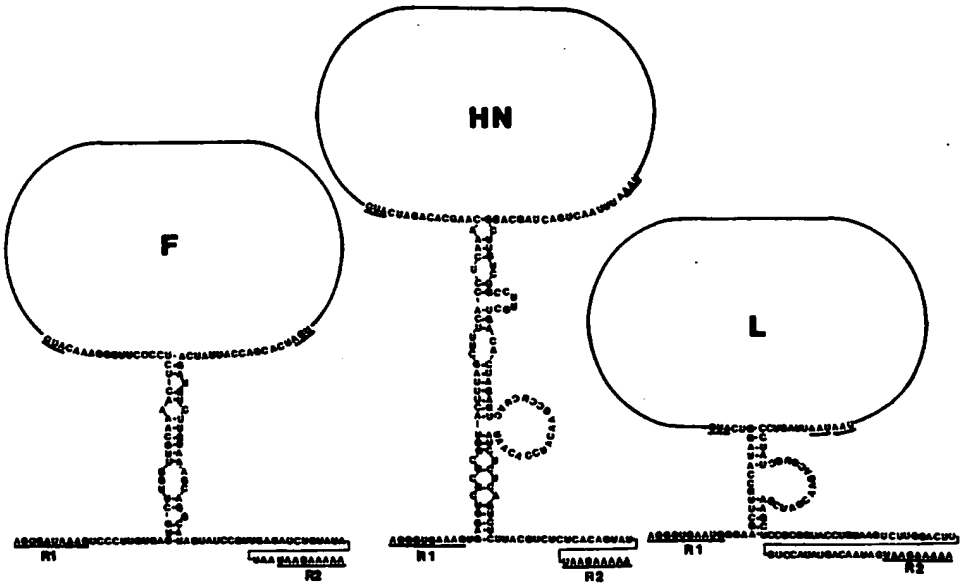


Fig. 6. Proposed secondary structures of the mRNAs for the F, HN and L proteins. The initiation and termination codons are underlined. R1 and R2 are the consensus sequences.

F protein

```
        10         20         30         40         50         60         70         80
MTAYIQRSQC ISTSLLVVLT TLVSCQIPRD RLSNIGVIVD EGKSLKIAGS HESRYIVLSL VPGVDFENGC GTAQVIQYKS   80
LLNRLLIPLR DALDLQEALI TVINDTFQNA GAPQSRFPGA VIGTIALGVA TSAQITAGIA LAEAREAKRD IALIKESMTK  160
THKSIELLQN AVGEQILALK TLQDFVNDEI KPAISELGCE TAALRLGIKL TQHYSELLTA PGSNPGTIGE KSLTLQALSS  240
LYSANITEIM TTIRTGQSNI SDVIYTEQIK GTVIDVDLER YMVTLSVKIP ILSEVPGVLI HKASSISYNI DGEEWYVTVP  320
SHILSRASFL GGADITDCVE SRLTYICPRD PAQLIPDSQQ KCILGDTTRC PVTKVVDSLI PKFAFVNGGV VANCIASTCT  400
CGTGRRPISQ DRSKGVVFLT HDNCGLIGVN GVELYANRRG HDATWGVQNL TVGPAIAIRP VDISLWLADA TNFLQDSKAE  480
LEKARKILSE VGRWYNSRET VITIIVVMVV ILVVIIVIII VLYRLRRSML MGNPDDRIPR DTYTLEPKIR HMYTMGGFDA  560
MAEKR                (565)
```

HN protein

```
        10         20         30         40         50         60         70         80
MDGDRGKRDS YWSTSPSGST TKPASGWERS SKADTWLLIL SFTQWALSIA TVIICIIISA RQGYSMKEYS MTVEALMNSS   80
REVKESLTSL IRQEVIARAV NIQSSVQTGI PVLLNKNSRD VIQMIDKSCS RQELTQHCES TIAVHHADGI APLEPHSFWR  160
CPVGEPYLSS DPEISLLPGP SLLSGSTTIS GCVRLPSLSI GEAIYAYSSN LITQGCADIG KSYQVLQLGY ISLNSDMFPD  240
LNPVVSHTYD INDNRKSCSV VATGTRGYQL CSMPTVDERT DYSSDGIEDL VLDVLDLKGR TKSHRYRNSE VDLDHPFSAL  320
YPSVGNGIAT EGSLIFLGYG GLTTPLQGDT KCRTQGCQQV SQDTCNEALK ITWLGGKQVV SVIIQVNDYL SERPKIRVTT  400
IPITQNYLGA EGRLLKLGDR VYIYTRSSGW HSQLQIGVLD VSHPLTINWT PHEALSRPGN KECNWYNKCP KECISGVYTD  480
AYPLSPDAAN VATVTLYANT SRVNPTIMYS NTTNIINMLR IKDVQLEAAY TTTSCITHFG KGYCFHIIEI NQKSLNTLQP  560
MLFKTSIPKL CKAES      (575)
```

L protein

```
        10         20         30         40         50         60         70         80
MDGQESSQNP SDILYPECHL NSPIVRGKIA QLHVLLDVNQ PYRLKDDSII NITKHKIRNG GLSPRQIKIR SLGKALQRTI   80
KDLDRYTFEP YPTYSQELLR LDIPEICDKI RSVFAVSDRL TRELSSGFQD LWLNIFKQLG NIEGREGYDP LQDIGTIPEI  160
TDKYSRNRWY RPFLTWFSIK YDMRWMQKTR PGGPLDTSNS HNLLECKSYT LVTYGDLVMI LNKLTLTGYI LTPELVLMYC  240
DVVEGRWNMS AAGHLDKKSI GITSKGEELW ELVDSLFSSL GEEIYNVIAL LEPLSLALIQ LNDPVIPLRG AFMRHVLTEL  320
QTVLTSRDVY TDAEADTIVE SLLAIFHGTS IDEKAEIFSF FRTFGHPSLE AVTAADKVRA HMYAQKAIKL KTLYECHAVF  400
CTIIINGYRE RHGGQWPPCD FPDHVCLELR NAQGSNTAIS YECAVDNYTS FIGFKFRKFI EPQLDEDLTI YMKDKALSPR  480
KEAWDSVYPD SNLYYKAPES EETRRLIEVF INDENFNPEE IINYVESGDW LKDEEFNISY SLKEKEIKQE GRLFAKMTYK  560
MRAVQVLAET LLAKGIGELF RENGMVKGEI DLLKRLTTLS VSGVPRTDSV YNNSKSSEKR NEGMENKNSG GYWDEKKRSR  640
HEFKATDSST DGYETLSCFL TTDLKKYCLN WRFESTALFG QRCNEIPGFK TPFNWMHPVL ERCTIYVGDP YCPVADRMHR  720
QLQDHADSGI FIHNPRGGIE GYCQKLWTLI SISAIHLAAV RVGVRVSAMV QGDNQAIAVT SRVPVAQTYK QKKNHVYEEI  800
TKYFGALRHV MFDVGHELKL NETIISSKMF VYSKRIYYDG KILPQCLKAL TKCVFWSETL VDENRSACSN ISTSIAKAIE  880
NGYSPILGYC IALYKTCQQV CISLGMTINP TISPTVRDQY FKGKNWLRCA VLIPANVGGF NYMSTSRCFV RNIGDPAVAA  960
LADLKRFIRA DLLDKQVLYR VMNQEPGDSS FLDWASDPYS CNLPHSQSIT TIIKNITARS VLQESPNPLL SGLFTETSGE 1040
EDLNLASFLM DRKVILPRVA HEILGNSLTG VREAIAGMLD TTKSLVRASV RKGGLSYGIL RRLVNYDLLQ YETLTRTLRK 1120
PVKDNIEYEY MCSVELAVGL RQKMWIHLTY GRPIHGLETP DPLELLRGIF IEGSEVCKLC RSEGADPIYT WFYLPDNIDL 1200
DTLTNGCPAI RIPYFGSATD ERSEAQLGYV RNLSKPAKAA IRIAMVYTWA YGTDEISWME AALIAQTRAN LSLENLKLLT 1280
PVSTSTNLSH RLKDTATQMK FSSATLVRAS RFITISNDNM ALKEAGESKD TNLVYQQIML TGLSLFEFNM RYKKGSLGKP 1360
LILHLHLNNG CCIMESPQEA NIPPRSTLDL EITQENNKLI YDPDPLKDVD LELFSKVRDV VHTVDMTYWS DDEVIRATSI 1440
CTAMTIADTM SQLDRDNLKE MIALVNDDDV NSLITEFMVI DVPLPCSTPG GILVNQFAYS LYGLNIRGRE EIWGHVVRIL 1520
KDTSHAVLKV LSNALSHPKI FKRFWNAGVV EPVYGPNLSW QDKILLALSV CEYSVDLFMH DMQGGVPLEI FICDNDPDVA 1600
DMRRSSFLAR HLAYLCSLAE ISRDGPRLES MNSLERLESL KSYLELTFLD DPVLRYSQLT GLVIKVFPST LTYIRKSSIK 1680
VLRTRGIGVP EVLEDWDPEA DNALLDGIAA EIQQNIPLGH QTRAPFWGLR VSKSQVLRLR GYKEITRGEI GRSGVGLTLP 1760
FDGRYLSHQL RLFGINSTSC LKALELTYLL SPLVDKDKDR LYLGEGAGAM LSCYDATLGP CINYYNSGVY SCDVWGQREL 1840
NIYPAEVALV GKKLNNVTSL GQRVKVLFNG NPGSTWIGND ECEALIWNEL QNSSIGLVHC DMEGGDHKDD QVVLHEHYSV 1920
IRIAYLVGDR DVVLISKIAP RLGTDWTRQL SLYLRYWDEV NLIVLKTSNP ASTEMYLLSR HPKSDIIEDS KTVLASLLPL 2000
SKEDSIKIEK WILIEKAKAH EWVTRELREG SSSSGMLRPY HQALQTPGFE PNLYKLSRDP LSTHNIADTH NCNIAFNRVL 2080
KDTIFEWARI TESDKRLKLT GKYDLYPVRD SGKLKTISRR LVLSWISLSM STRLVTGSFP DQKFEARLQL GIVSLSSREI 2160
RNLRVITKTL LDRFEDIIHS ITYRFLTKEI KILMKILGAV KMFGARQNEY TTVIDDGSLG DIEPYDSS      (2228)
```

here the primary structure of the entire Sendai virus genome, which is schematically illustrated in Fig. 5. As a whole, 99.17% of the Sendai virus genome is transcribed into mRNA and 93.63% is translated into proteins, indicating that the structure of the genome is utilized quite efficiently.

It is noteworthy that an open reading frame corresponding to 249 amino acids was detected in the genome sense strand within the L gene region (from nucleotide position 9,588 to 8,842), which is longer than that of the Sendai virus C protein, and the only one long open reading frame capable of coding for more than 150 amino acids in the genome strand. However, this frame is not flanked by R1 and R2, nor could a single stranded cDNA probe complementary to this open reading frame detect any subgenomic transcript from the infected cells, either poly (A) plus or poly (A) minus (data not shown). From these observations, it seems unlikely that this open reading frame is transcribed, although further studies should be carried out before a definite conclusion is drawn.

Proposed structures of mRNAs for the F, HN and L proteins

From the nucleotide sequence of the genome RNA, the nucleotide sequences of mRNAs for the F, HN and L proteins were deduced. On detailed examination of these sequences, it was found that in every mRNA, a part of the 5' noncoding sequence was complementary to that of the 3' noncoding sequence, suggesting that these ends might form a double-stranded structure, which gives the mRNA a panhandle structure (Fig. 6). These secondary structures seems to be fairly stable on the basis of the free energy levels (30).

Characteristics of the F, HN and L gene products

The amino acid sequences of the F, HN and L gene products are shown in Fig. 7. The deduced amino acid sequence of the F protein indicates that the F protein is highly hydrophobic overall. As we reported previously (4), a signal peptide of 24 to 27 amino acids was detected in its N terminus, and the cleavage site for the F1 and F2 proteins was assumed to be the arginine residue at position 116. The most hydrophobic region of this protein is located near its C terminus (from amino acid position 500 to 523), which is followed by a hydrophilic region of 42 amino acids. The F protein of Sendai virus has been reported to penetrate the viral envelope, leaving an at least 3K portion exposed to the inside of the envelope (31), which is similar to

Fig. 7. The predicted amino acid sequences of the F, HN and L proteins. The putative N-linked carbohydrate attachment sites are boxed. The underlining indicates the most hydrophobic regions.

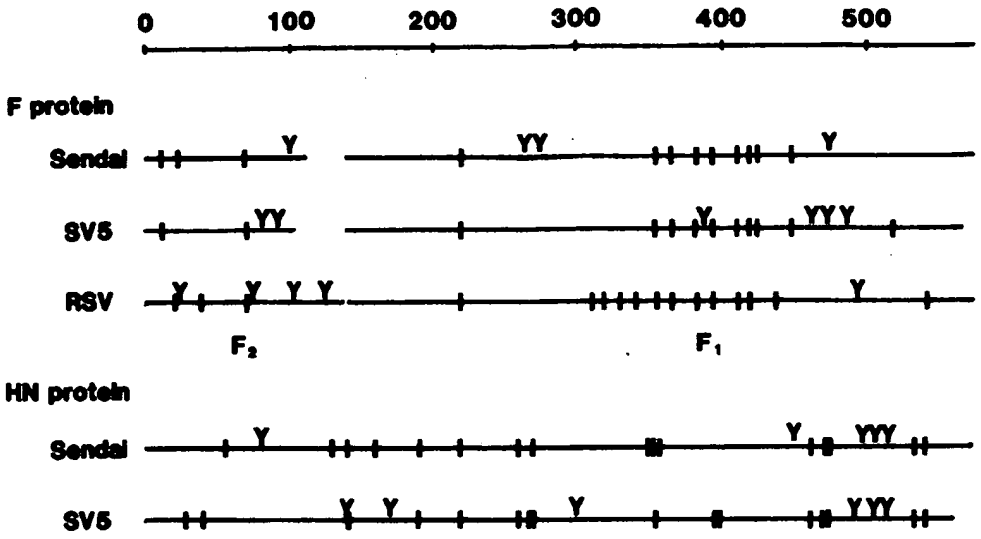


Fig. 8. Locations of the cysteine residues (vertical bars) and the putative N-linked carbohydrate attachment sites (Y) within the F proteins of Sendai virus, simian virus 5 (SV5) and respiratory syncytial virus (RSV), and the HN proteins of Sendai virus and SV5. To align the N termini of the F1 subunits, small gaps are left between the F2 and F1 subunits of Sendai virus and SV5.

in the case of hemagglutinin of influenza virus (32). Thus, it is probable that the hydrophobic region near the C terminus may anchor the F protein within the viral envelope and the very C terminal hydrophilic region may be located at the inner surface of the viral envelope, interacting with the viral M protein. As shown in Fig.8, the F protein has 12 cysteine residues, of which 2 are found in the signal peptide, 1 in the F2 portion and 9 in the F1 portion. Eight of the 9 cysteine residues in the F1 portion are clustered in its middle part, from position 338 to 424. Since the results of secondary structure analysis (33) indicated many reverse turns within the cysteine-rich portion of the F1 protein, this cluster may stabilize the tertiary structure of the F protein by forming intramolecular disulphide bonds. We could detect four putative N-linked carbohydrate attachment sites (Asn-X-Ser or Asn-X-Thr) (34) in the F protein, i.e. from position 104 to 106, 245 to 247, 259 to 261, and 449 to 451, respectively, which is in good agreement with the results reported by Kohama et al. (35) showing that the F protein of Sendai virus has four N-linked carbohydrate chains.

The most hydrophobic region of the HN protein is, in contrast to in the F protein, located near the N terminus, i.e. from position 36 to 58, which is preceded by a hydrophilic domain of 35 amino acids. This structure

resembles that of influenza virus neuraminidase (36,37), in which the hydrophobic region near the N terminus serves as an signal for membrane translocation and as an anchor in the membrane. Thus, it is plausible that this hydrophobic region near the N terminus of the HN protein may act as a signal and an anchor, and the preceding hydrophilic region of 35 amino acids is exposed to the inside of the viral envelope, since the HN protein was reported to penetrate the viral envelope and the molecular weight of the portion exposed to the inside of the envelope was estimated to be at least 2K (31). Within the deduced amino acid sequence of the HN protein, we found five N-linked carbohydrate attachment sites, i.e. 77 to 79, 448 to 450, 499 to 501, 504 to 506, and 511 to 513, which is in accordance with previous suggestion that the HN protein of Sendai virus has at least four N-linked carbohydrate chains (35).

Analyses of the deduced amino acid sequence of the L gene product provided us with little special information as to the structure of the L protein or its functional domains, except that Ser-Asp-Asp found from position 1,429 to 1,431 or Leu-Asp-Asp from 1,649 to 1,651 might be the active site of RNA synthesis as in the case of reverse transcriptases of retroviruses and RNA polymerases of picornaviruses (38). It is interesting to note that there is a possibility that another L gene product might be present which starts from the sixth AUG codon in mRNA, corresponding to the methionine at position 249 of the L protein or nucleotide position 9,299 to 9,301 with a calculated molecular weight of 224,005, since this codon is preceded by A 3 nucleotides upstream while the first five AUG codons are preceded by G or U, and according to Kozak (39), A might be preferentially recognized by ribosomes.

Comparison of the Z and Harris strains as to the F and HN genes

Recently, Blumberg et al. (8) reported the complete nucleotide sequence of the F gene of the Harris strain of Sendai virus as well as the sequence of the tripeptide of the N terminus of the F2 protein purified from virions of the same strain. On comparison of the results presented in this paper with theirs neither insertion nor deletion could be detected, and 98.63% of the nucleotides and 97.88% of the amino acids were found to be conserved between these two strains. The observation that the N terminus of the F2 protein of the Harris strain was glutamic acid at amino acid position 26 is in good agreement with our previous prediction as to the cleavage site of the signal peptide (4), thus we concluded that the peptide from 1 to 25 is the signal peptide and that from 26 to 116 is the F2 protein.

During preparation of this manuscript, the nucleotide sequences of the HN genes of the Harris strain (40) and Z strain (41) of Sendai virus were also presented. Comparing our present results for the Z strain with those for the Harris strain, we found that the latter is longer than the former by one amino acid residue, namely, the genome of the latter strain has an additional -AAA- between nucleotide positions 8,288 and 8,289 of that of the former, which led to an insertion of a serine residue. This insertion seems to make no significant difference as to the structure of the HN protein between these strains. Except for this insertion, most of the nucleotide sequence as well as the amino acid sequence was conserved between these two strains, giving 98.52% and 97.57% homology, respectively. On comparison of our results and those presented by Miura et al. (41) on the HN protein of the Z strain, four substitutions of amino acid residues were found while neither insertion nor deletion was detected.

Comparison of Sendai virus and SV5 as to the F and HN proteins

The nucleotide sequences of the F and HN genes of SV5, another paramyxovirus, were also reported recently (12,13). When the amino acid sequence of the F protein of the Sendai virus Z strain described above was aligned with that of SV5 to give minimal gaps for comparison, 133 amino acids of the F proteins were found to coincide with each other, but the overall homology was estimated to be only 23.5%. Interestingly, however, certain portions of the proteins show more than 50% homology, which were found from amino acid position 116 to 135 and from 458 to 477 of the F protein of Sendai virus (Fig. 9). This suggests the importance of these sequences for the function of the protein, since the N terminal portion of the F1 protein seemed to act as a functional domain during membrane fusion (42). It is noteworthy that the distribution of cysteine residues within the F proteins was well conserved between these two viruses. Both the F protein of Sendai virus and that of SV5 have 12 cysteine residues, out of which 10 could be aligned at the same positions, 1 in the F2 portion and 9 in the F1 portion, as shown in Fig. 8. The eight cysteine residues clustered in the middle part of the F1 protein of Sendai virus are all conserved at the corresponding positions of the F protein of SV5. In spite of these similarities, however, there is no indication that the carbohydrate attachment sites are distributed similarly in the F proteins of the two viruses.

When the amino acid sequences of the HN proteins of these viruses were aligned, 138 amino acids coincided and the overall homology was about 24%.

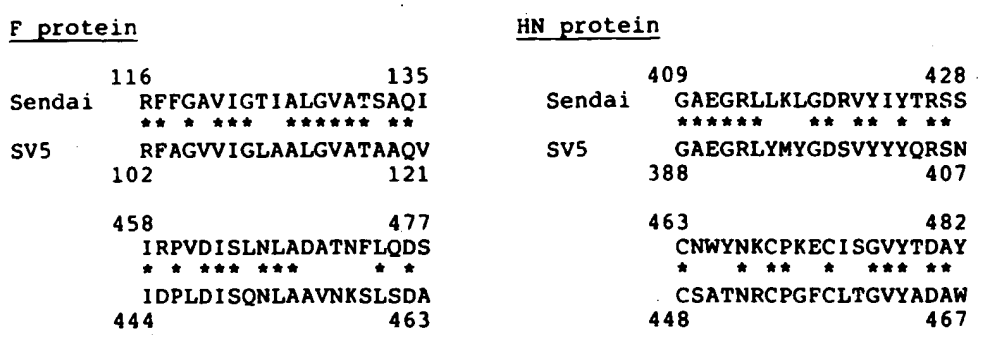


Fig. 9. Highly conserved regions of the F and HN proteins of Sendai virus and SV5. Numbers indicate the amino acid positions. Asterisks indicate the identity of amino acid residues.

As in the case of the F proteins, however, highly conserved portions were found from amino acid position 409 to 428 and from amino 463 to 483 (Fig.9), which may be included in the active sites for hemagglutinin and neuraminidase. Similarities could also be found between these two viruses in the locations of the cysteine residues in the HN proteins and those of carbohydrate attachment sites, three of which were found in the C terminal regions of the proteins.

DISCUSSION

At the beginning of our work concerning the determination of the nucleotide sequence of the Sendai virus genome RNA, we obtained relatively long cDNAs, of about 3,000 to 4,000 nucleotides in length, starting from its 3' end, which could be sequenced satisfactorily (3). However, attempts to elongate these cDNAs by the primer extension method generally only yielded short cDNA clones of about 600 to 1,000 nucleotides in length (4), which greatly hampered the determination of the entire nucleotide sequence of the Sendai virus genome RNA. Thus, we decided to adopt a new cDNA cloning strategy, which involved starting cDNA synthesis from multiple sites in the genome RNA in combination with the cloning method of Okayama and Berg (16). It was nesessary for this purpose to cut the genome RNA into a few fragments and to add a poly(A)-tail to the resulting fragments. We found that this could be achieved by using an excess amount of poly A polymerase (P-L Biochemicals, lot no. 206-7) during the polyadenylylation reaction of the genome RNA, since we had found that a trace amount of ribonuclease activity contaminated the poly A polymerase preparation. Thus, we succeeded in establishing a set of cDNA copies that completely covered the Sendai virus

genome RNA, and could determine the whole sequence of the genome RNA of 15,383 nucleotides, which revealed that the gene structure of the Sendai virus is 3'-NP-P+C-M-F-HN-L-5'.

One of the characteristic features of the genome is that each gene is flanked by consensus sequences at both ends, that is, R1 at the 3' end and R2 at the 5' end. Since R1 shows minor variations from gene to gene, i.e. UCCCAGUUUC for the NP gene (3), UCCCACUUUC for the P+C (3), M (4) and HN genes, UCCCUAUUUC for the F gene (4), and UCCCACUUAC for the L gene, its common structure was deduced to be UCCC-A/U-C/G/A-UU-U/A-C or UCCCNNUUNC. On the other hand, R2 was found to be AUUCUUUUU for all genes. It is highly possible that R1 is the recognition sequence for viral RNA polymerase, minor differences in which may play a role in the control of the expression of each gene, while R2 is a polyadenylation signal. Consensus sequences similar to R1 and R2 were also reported for non-segmented negative stranded RNA viruses, i.e. VSV (43,44), respiratory syncytial virus (RSV) (45) and measles virus (46), suggesting their common importance in the transcription and/or replication process of these viruses. It is interesting to note that the sequence, GAA or GGG, which was found between two adjacent genes (or between R2 and R1) and thought to be an intergenic sequence (3,4), was detected after R2 of the L gene. This strongly indicates that R2 together with this trinucleotide may constitute a signal sequence for the termination of transcription as well as for polyadenylation. The finding that the 12 nucleotides of the very 5' end of the genome are complementary to the 12 nucleotides of the very 3' end of the genome is very important because it supports the prediction that the genome would form a very stable panhandle structure, which will provide the signal sequences for recognition by viral RNA polymerase and for association between the RNA and nucleocapsid proteins (47).

In VSV, it has been reported that about 50 nucleotides of the very 3' end of both the genome and antigenome RNA are transcribed to small RNAs designated as plus and minus leader RNAs, respectively (2,48). In Sendai virus, however, plus leader RNA but not minus leader RNA was detected in infected cells (2). Accordingly, it is of interest to investigate whether the 3' terminal 54 nucleotides of the Sendai virus antigenome, which is complementary to the 5' terminal 54 nucleotides of the genome RNA, can be transcribed to produce minus leader RNA, and to determine the function of minus leader RNA.

It is noteworthy that a stable secondary structure could be formed

within the non-coding portions of all the mRNAs for the F, HN and L proteins, giving each mRNA a panhandle structure, and similar structures are also possible for the construction of mRNAs for the NP, P+C and M genes (not shown). This type of secondary structure has been proposed for the mRNA of gene 10 of human rota virus (49), although the panhandle structure involves coding sequences in this case. The model presented in the present paper is of special interest, since the panhandle is constructed from only non-coding sequences, leaving the initiation as well as the termination codon within the single-stranded loop structure. According to this model, this secondary structure of the mRNA may be very important for its translation, because ribosomes may select the first AUG in the loop as the initiation codon, and bind directly to it with the aid of the initiation factors. In this regard, it is interesting to note that two forms of the secondary structure are possible for the construction of the mRNA of the P+C gene, which has two open reading frames that overlap (3). In one form, the initiation codon as well as the termination codon for both the P and C proteins are present within the loop structure, whereas in the other form, both the initation and termination codons for the P protein are buried in the stem structure, while those for the C protein remain in the loop structure. Details of these structures will be published elsewhere.

Recently, the nucleotide sequence of the F gene of respiratory syncytial virus (RSV) was reported (45,50). As expected from the fact that this virus is classified as a pneumovirus different from paramyxoviruses (51), there is little homology between the deduced amino acid sequence of this gene and that of the Sendai virus F gene. It is noteworthy, however, that the F protein of RSV also has a cluster of cysteine residues in the middle portion of the F1 protein (Fig. 8) as the F proteins of Sendai virus and SV5 do, indicating that this structure may be very important in the determination of the tertiary structure of biologically active F proteins.

The L protein of paramyxoviruses is expected to exhibit multifunctional activities as to viral transcription and replication, including the initiation, elongation, termination, polyadenylation, methylation and capping reactions, as in the case of the L protein of VSV. However, we could not find any significant homology in the amino acid sequence between the L gene product of Sendai virus and that of VSV (52), and it is too early to infer the functional domains of L proteins, and it is necessary to have further information on the structures of the L proteins of other

paramyxovirus. Therefore, we have started analyzing the L gene of bovine parainfluenza type 3 virus.

ACKNOWLEDGEMENT

We wish to express our thanks to Dr. A. Nomoto for his helpful discussions. This work was supported by grants from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan.

*To whom correspondence should be addressed

REFERENCES

1. Lamb,R.A. and Choppin,P.W. (1978) Virology 84, 469-478.
2. Leppert,M., Rittenhouse,L. Perraut,J., Summers,D.F. and Kolakofsky,D. (1979) Cell 18, 735-747.
3. Shioda,T., Hidaka,Y., Kanda,T., Shibuta,H., Nomoto,A. and Iwasaki,K. (1983) Nucleic Acids Res. 11, 7317-7333.
4. Hidaka,Y., Kanda,T., Iwasaki,K., Nomoto,A., Shioda,T. and Shibuta,H. (1984) Nucleic Acids Res. 12, 7965-7972.
5. Giorgi,C., Blumberg,B.M. and Kolakofsky,D. (1983) Cell 35, 829-936.
6. Blumberg,B.M., Giorge,C., Rose,K. and Kolakofsky,D. (1984) J.gen.Virol. 65, 769-779.
7. Blumberg,B.M., Rose,K., Simona,M.G., Roux,L., Giorgi,C. and Kolakofsky,D. (1984) J.Virol. 52, 656-663.
8. Blumberg,B.M., Gorgi,C., Rose,K. and Kolakofsky,D. (1985) J.gen.Virol. 66, 317-331.
9. Morgan,E.M., Re,G.G. and Kingsbury,D.W. (1984) Virolgy 135, 279-287.
10. Dowling,P.C., Giorgi,C., Roux,L., Dethlesen,L.A., Galantowitz,M.E., Blumberg,B.M. and Kolakofsky,D. (1983) Proc.Natl.Acad.Sci. USA. 80, 5213-5216.
11. Glazier,K., Raghow,R. and Kingsbury,D.W. (1977) J.Virol. 21, 863-871.
12. Paterson,R.G., Harris,T.J.R. and Lamb,R.A. (1984) Proc.Natl.Acad.Sci. USA. 81, 6706-6710.
13. Hiebert,S.W., Paterson,R.G. and Lamb,R.A. (1985) J.Virol. 54, 1-6.
14. Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) Biochmistry 18, 5294-5299.
15. Inokuchi,Y. Hirashima,A. and Watanabe,I. (1982) J.Mol.Biol. 158, 711-730.
16. Okayama,H. and Berg,P. (1982) Mol.Cel.Biol. 2, 161-170.
17. Boliver,F. and Backman,K. (1979) Methods Enzymol. 68, 245-267.
18. Messing,J. (1983) Methods Enzymol. 101, 20-78.
19. Sanger,F., Nicklen,S. and Coulson,A.R. (1977) Proc.Natl.Acad.Sci. USA. 74, 5463-5467.
20. Grunstein,M. and Hogness,D.S. (1975) Proc.Natl.Acad.Sci. USA. 72, 3961-3965.
21. Wahl,G.M., Stern,M. and Stark,G.R. (1979) Proc.Natl.Acad.Sci. USA. 76, 3683-3687.
22. Maxam,A.M. and Gilbert,W. (1980) Methods Enzymol. 65, 499-560.
23. Rigby,P.W.J., Deckman,M., Rhodes,C. and Berg,P. (1977) J.Mol.Biol. 113, 237-259.
24. Re,G.G., Gupta,K.C. and Kingsbury,D.W. (1983) Virology 130, 390-396.

25. Kolakofsky,D., Boy de la Tour,E. and Delius,H. (1974) J.Virol. 13, 261-268.
26. Shibuta,H., Kanda,T., Adachi,A. and Yogo,Y. (1979) Microbiol. Immunol. 23, 617-628.
27. Nakamura,K., Homma,M. and Compans,R.W. (1982) Virology 119, 474-487.
28. Hsu, M.-C. and Choppin,P.W. (1984) Proc.Natl.Acad.Sci.USA. 81, 7732-7736.
29. Lamb,R.A., Mahy,B.W.J. and Choppin,P.W. (1976) Virology 69, 116-131.
30. Tinoco Jr.I., Borer,P.N., Dengler,B., Levine,M.D., Uhlenbeck,O.C., Chorothers,D.M. and Gralla,J. (1973) Nature New Biol. 246, 40-41.
31. Lyle,D.S. (1979) Proc.Natl.Acad.Sci.USA. 76, 5621-5625.
32. Gething,M.J., Bye,J., Skehel,J. and Waterfield,M. (1980) Nature 287, 301-306.
33. Chou,P.Y. and Fasman,G.D. (1978) Adv.Enzymology 47, 45-148.
34. Neuberger,A., Gottschalk,A., Marshall,R.D. and Spiro,R.G. (1972) in The Glycoproteins: Their composition, structure and function, Gottschalk,A. Ed., pp. 450-490, Elsevier, Amsterdam.
35. Kohama,T., Shimizu,K. and Ishida,N. (1978) Virology 90, 226-234.
36. Blok,J., Air,G.M., Laver,W.G., Ward,C.W., Lilley,G.G., Woods,E.F., Roxburgh,C.M. and Inglis,A.S. (1982) Virology 119, 109-121.
37. Bos,T.J., Davis,A.R. and Nayak,D.P. (1984) Proc.Natl.Acad.Sci.USA. 81, 2327-2331.
38. Kamer,G. and Agros,P. (1984) Nucleic Acids Res. 12, 7269-7282.
39. Kozak,M. (1984) Nature 308, 241-246.
40. Blumberg,B.M., Giorgi,C., Roux,L., Ramaswamy,R., Dowling,P., Chollet,A. and Kolakofsky,D. (1985) Cell 41, 269-278.
41. Miura,N., Nakatani,Y., Ishimura,M., Uchida.T. and Okada,Y. (1985) FEBS lett. 188, 112-116.
42. Richardson,C.D., Sceid,A. and Choppin,P.W. (1980) Virology 105, 205-222.
43. Rowlands,D.J. (1979) Proc.Natl.Acad.Sci. USA. 76, 4793-4797.
44. Rose,J.K. (1980) Cell 19, 415-421.
45. Collins,P.L., Huang,Y.T. Wertz,G.W. (1984) Proc.Natl.Acad.Sci. 81, 7683-7687.
46. Bellini,W.J., Englund,G., Rozenblatt,S., Arnheiter,H. and Richardson,C.D. (1985) J.Virol. 53, 908-919.
47. Blumberg,B.M., Giorgi,C. and Kolakofsky,D. (1983) Cell 32, 559-567.
48. Colonno,R.J. and Banerjee,A.K. (1976) Cell 8, 197-204.
49. Okada,Y., Richardson,M.A., Ikegami,N., Nomoto,A. and Furuichi,Y. (1984) J.Virol. 51, 856-859.
50. Elango,N., Satake,M., Coligan,J.E., Norrby,E., Camargo,E. and Venkatesen,S. (1985) Nucleic Acids Res. 13, 1559-1574.
51. Matthews,R.E.F. (1982) Intervirology 17, 1-200.
52. Schubert,M., Harmison,G.G. and Meier,E. (1984) J.Virol. 51, 505-514.

S. Martinek
2429

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/724,388	11/28/2000	Jin Hong	7682-051-999	8169

20583 7590 11/19/2002

PENNIE AND EDMONDS
1155 AVENUE OF THE AMERICAS
NEW YORK, NY 100362711

S. Martinek

RECD
NOV 2 6 2002
Pennie & Edmonds
O.K. for filing

EXAMINER
LUCAS, ZACHARIAH

ART UNIT	PAPER NUMBER
1648	12

DATE MAILED: 11/19/2002

Response to Restriction Req. 12/19/02

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No. 09/724,388	Applicant(s) HONG ET AL.	
	Examiner Zachariah Lucas	Art Unit 1648	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *28 November 2000* .

2a)☐ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *7-16* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *7-16* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other:

DETAILED ACTION

Election/Restrictions

1. Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 7-12, drawn to recombinant paramyxoviridae viruses, classified in class 435, subclass 235.1.

II. Claims 13-16, drawn to methods of making recombinant negative stranded RNA viruses, classified in class 435, subclass 239.

For each of Groups I and II above, restriction to one of the following is also required under 35 USC 121. Therefore, election is required of one of Groups I-II, and, to one of inventions (A)-(B). The inventions of Groups A and B represent the elected invention wherein the recombinant virus is (A) a parainfluenza virus or (B) a respiratory syncytial virus.

The inventions are distinct, each from the others, for the following reasons:

2. The inventions of Groups (A) and (B) are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different modes of operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case, the different inventions each relate to a different genus of recombinant virus. Each of these viruses contain different genetic sequences and different proteins, and have different structures. Due to these differences, and because the inventions are not disclosed as usable together, the inventions of these Groups are distinct.

3. Inventions I and II are related as process of making and product made. The inventions are distinct if either or both of the following can be shown: (1) that the process as claimed can be used to make other and materially different product or (2) that the product as claimed can be made by another and materially different process (MPEP § 806.05(f)). In the instant case, the

methods may be used not only to make recombinant paramyxoviridae viruses, but also to make other recombinant negative stranded viruses. Because the methods of Group II may be used to make other recombinant viruses than those claimed, the methods of Group II are distinct from those of Group I.

Examiner's Notes

4. The examiner would like to bring the following to the applicant's attention. Claim 8, added in Amend. A, filed Feb. 28, 2001, is a dependant claim that depends from itself. This renders the claim indefinite. Although no rejection is being made at this time, it is suggested that the claim be corrected if the applicant elects a Group of inventions comprising this claim.

5. It is here noted that some of the restrictions requirements made above fall within the scope of PTO Linking claim practice. In accordance with this practice as described in MPEP 809.03, linking claims will be considered with the elected invention. If the elected invention is found allowable, the linking claim will also be examined. If no substantive rejection is found for the linking claim, the restriction among the Groups it comprises will be withdrawn. The linking claims have been including in the Groupings with the linked inventions.

6. Applicant's attention is hereby directed to the following is a recitation of M.P.E.P. §821.04 regarding the restriction of claims to a product and processes of using the product, Rejoinder:

> Where product and process claims drawn to independent and distinct inventions are presented in the same application, applicant may be called upon under 35 U.S.C. 121 to elect claims to either the product or process. See MPEP § 806.05(f) and § 806.05(h). The claims to the nonelected invention will be withdrawn from further consideration under 37 CFR 1.142. See MPEP § 809.02© and § 821 through § 821.03. However, if applicant elects claims directed to the product, and a product claim is subsequently found allowable, withdrawn process claims which depend from or otherwise include all the limitations of the allowable product claim will be rejoined.

> Where product and process claims are presented in a single application and that application qualifies under the transitional restriction practice pursuant to 37 CFR 1.129(b), applicant may either (1) elect the invention to be searched and examined and pay the fee set forth in 37 CFR 1.17(s) and have the additional inventions searched and examined under 37 CFR 1.129(b)(2), or (2) elect the invention to be searched and examined and not pay the additional fee (37 CFR 1.129(b)(3)). Where no additional fee is paid, if the elected invention is directed to the product and the claims directed to the product are subsequently found patentable, process claims which either depend from or include all the limitations of the allowable product will be rejoined. If applicant chooses to pay the fees to have the additional inventions searched and examined pursuant to 37 CFR 1.129(b)(2), even if the product is found allowable, applicant would not be entitled to a refund of the fees paid under 37 CFR 1.129(b) by arguing that the process claims could have been rejoined. 37 CFR 1.26 states that "[m]oney paid by actual mistake or in excess will be refunded, but a mere change of purpose after the payment of money...will not entitle a party to demand such a return..." The fees paid under 37 CFR 1.129(b) were not paid by actual mistake nor paid in excess, therefore, applicant would not be entitled to a refund.
>
> In the event of rejoinder, the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104 - 1.106. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103, and 112. If the application containing the rejoined claims is not in condition for allowance, the subsequent Office action may be made final, or, if the application was already under final rejection, the next Office action may be an advisory action.

The following is a recitation from paragraph five, "Guidance on Treatment of Product and Process Claims in light of *In re Ochiai, In re Brouwer* and 35 U.S.C. §103(b)" (1184 TMOG 86(March 26, 1996)):

> "However, in the case of an elected product claim, rejoinder will be permitted when a product claim is found allowable and the withdrawn process claim **depends from or otherwise includes all the limitations of** an allowed product claim. Withdrawn process claims not commensurate in scope with an allowed product claim will not be rejoined." (emphasis added)

In accordance with M.P.E.P. §821.04 and In re Ochiai, 71 F.3d 1565, 37 USPQ 1127 (Fed. Cir. 1995), rejoinder of product claims with process claims commensurate in scope with the allowed product claims will occur following a finding that the product claims are allowable. Until, such time, a restriction between product claims and process claims is deemed proper. Additionally, in order to retain the right to rejoinder in accordance with the above policy, Applicant is advised that the process claims should be amended during prosecution to maintain either dependency on the product claims or to otherwise include the limitations of the product claims. **Failure to do so may result in a loss of the right to rejoinder.**

7.

Conclusion

8. Because these inventions are distinct for the reasons given above, have different classifications, and because the literature and sequence searches required for any one of the groups is not required for the others, restriction for examination purposes as indicated is proper.

9. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

10. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Zachariah Lucas whose telephone number is 703-308-4240. The examiner can normally be reached on Monday-Friday, 8 am to 4:30 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Housel can be reached on 703-308-4027. The fax phone numbers for the organization where this application or proceeding is assigned are 703-308-4242 for regular communications and 703-872-9307 for After Final communications.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0196.

Z. Lucas
Patent Examiner
November 6, 2002

JAMES HOUSEL 11/18/02
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600